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**Zinc Accumulation and its Effects on Herbivory and
Competitive Ability, in Metallicolous Populations of *Rumex*
acetosa L.**

By

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A thesis submitted for the degree of

Doctor of Philosophy

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University of Sussex
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Declaration:

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another university for the award of any other degree.

Signature:.....

(Claudia Harflett).

Acknowledgements:

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Thesis Abstract:

**Zinc Accumulation and its Effects on Herbivory and Competitive Ability, in
Metallicolous Populations of *Rumex acetosa* L.**

The Elemental Defence Hypothesis, proposed by Boyd and Martens in 1992, suggests that high foliar metal concentrations deter herbivore feeding and this protection from herbivory may be one factor explaining the adaptive value of metal hyperaccumulation. However, lower foliar metal concentrations than those occurring in hyperaccumulators, may also confer this advantage, but the benefits of metal uptake by this group of plants (known as accumulators) has been relatively less-studied. Despite this potential advantage, metal accumulation is a relatively rare phenomenon, suggesting it may have costs as well as benefits.

A field survey of metallicolous populations of the hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. (recently renamed as *Noccaea caerulescens* (J. & C. Presl.) F. K. Meyer) (Brassicaceae) and the accumulator *Rumex acetosa* L. (Polygonaceae), found on zinc (Zn) contaminated mining sites, revealed between-population differences in chewing herbivore damage, in the efficiency with which they uptake soil Zn into their shoots (measured as the concentration factor), and the foliar Zn concentration of *T. caerulescens*. However, foliar Zn concentration was not correlated with damage within a species.

In a series of pot experiments using two populations of *R. acetosa*, the foliar Zn concentration was manipulated through the addition of Zn to the soil and through differences in Zn uptake rate between populations. This thesis investigated how these manipulations influenced herbivory by generalist *Helix aspersa* Müller (Helicidae), and how plant competitive ability (in terms of biomass) was determined by a combination of population identity, soil Zn concentration and presence of herbivores.

When two *R. acetosa* populations were grown under 1500 and 45,000 mg/kg soil Zn concentrations, population differences were found in shoot biomass and competitive ability. The outcomes of intra- compared with inter-population competition depended on soil Zn concentration. When herbivores were present, shoot damage was low, usually < 15% of foliage removed. Snail preference was dependent upon the interaction between population identity and soil Zn concentration, partially supporting the Elemental Defence Hypothesis.

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Chapter 1: General Introduction:

1.1 Chapter Overview:

Much of the research into plant-herbivore interactions has focused on plant defence strategies, especially the influence of carbon-based chemical defences (i.e. organic defence compounds, such as terpenes) (Spiteller, Sven Erik & Brian 2008). However the use of inorganic compounds such as metals, termed 'Elemental Defence' by Boyd & Martens (1992), is an alternative potential herbivore defence strategy. The Elemental Defence Hypothesis (EDH) suggests that high foliar metal concentrations are toxic to herbivores and can deter herbivory, leading to a selective advantage of higher foliar metal concentrations. Plants growing in habitats with elevated metal soil concentrations, termed 'metallicolous communities', are able to tolerate the toxic soil conditions and excessive movement of metals into their aerial parts. Some metallicolous plants, called 'hyperaccumulators', are able to actively transport metal ions into their shoots and store them at extraordinarily high levels, with no detrimental effects to the plant. The EDH, was first proposed by Boyd & Martens (1992) following their study of nickel (Ni) hyperaccumulators. However, metallicolous plant communities also contain many plant species with lower foliar metal concentrations than hyperaccumulators, but which are still highly elevated. These plants are known as 'accumulators' (Reeves & Baker 2000; Boyd 2007). Studies investigating the EDH have rarely investigated the ability of the metal concentrations in accumulator species to deter herbivory, termed 'defensive enhancement' (Boyd 2007), although there is some evidence to suggest that this may be the case (Coleman, Boyd & Eubanks 2005; Boyd 2007). Tolerating toxic concentrations of metals in the soil requires certain physiological adaptations, which can be considered as 'costly' to the plant's overall productivity and fitness (Baker 1987; Ernst, Schat & Verkleij 1990; Wu

1990; Harper, Smith & Macnair 1997a; Kazakou *et al.* 2008; Maestri *et al.* 2010).

Thus the soil metal concentration may influence the competitive ability of metal-tolerant plants as well as their defensive strategies. The use of metals as a defence and its impact on competitive ability under different herbivory regimes, are investigated in this thesis using metallicolous populations of the zinc (Zn) accumulator *Rumex acetosa* L. and hyperaccumulator *Thlaspi caerulescens* (recently renamed as *Noccaea caerulescens* (J. & C. Presl.) F. K. Meyer), from the Zn-rich soils of the Southern Pennines, England, and the generalist snail herbivore *Helix aspersa* Müller.

1.2 Plant Defence Strategies:

The biological and chemical interactions between plants and their herbivores are diverse and complex. Plants are susceptible to attack from a wide variety of specialist and generalist herbivores, reducing plant productivity and fecundity, and resulting in the evolution of both plant defences and animal adaptations to these defences over 410 million years of interactions (Johnson 2011). However, irrespective of any defences they have evolved, plants are generally poor quality food for herbivores because of their high carbon to nitrogen ratio, sometimes low water content, and toughness and indigestibility due to compounds such as lignin and cellulose (Hartley & Jones 1997). Plants also have a variety of chemical and physical defence strategies to counterattack the wide range of herbivore taxa and feeding guilds that they interact with (Strauss & Zangerl 2002) (but see Agrawal (2000)).

A huge variety of plant defence strategies have evolved across different plant families (Agrawal 2007) and a particular plant species may adopt primarily one type of strategy, resulting in a trade-off against the other strategies, though they

are not mutually exclusive (Agrawal & Fishbein 2006). There are three main strategies to dealing with herbivore attack:

1) Tolerance or Compensation, whereby the plant invests locally-abundant resources (either external or internal to the plant) into faster and greater re-growth (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999; Agrawal 2000; Endara & Coley 2010).

2) Avoidance, whereby unpredictability in a plant species allows the plants to evade herbivores, especially specialists which are able to disarm the constitutive chemical defences in place. This unpredictability can occur spatially (Feeny 1977; Endara & Coley 2010) or temporally (Pilson 2000).

3) Resistance through the use of chemical and physical defences (Strauss & Zangerl 2002). Resistance strategies can act in a 'direct' manner such as deterrence through taste and reducing herbivore performance and fecundity, or in an 'indirect' manner such as accommodating and signalling to natural enemies of the herbivores (Spiteller, Sven Erik & Brian 2008). This thesis focuses upon direct chemical defences, which are discussed further below.

1.2.1 *Direct Plant Defences:*

There are a huge variety of direct plant defences which can be divided into two main groups:

1) Physical defences such as thorns, trichomes and leaf toughness; thorns are especially effective against mammalian herbivores, and the latter two against invertebrate generalists (Hanley *et al.* 2007).

2) A complex array of **chemical defences**, usually formed from secondary metabolites and varying in their biosynthetic derivation, structure, chemical composition and concentration (Levin 1976; Harborne 1997; Strauss & Zangerl 2002; Spiteller, Sven Erik & Brian 2008). Organic chemical defences are those derived from the products of photosynthesis and hence they are predominantly made-up of carbon-based molecules. Examples of such secondary metabolites include terpenes and phenolic compounds.

Organic defence compounds have two major drawbacks – 1) cost of production and 2) detoxification by specialist herbivores. The synthesis of secondary compounds is derived from primary biochemical pathways, and can be seen as costly as the plant has to allocate resources away from other processes such as growth and reproduction (Levin 1976; Simms 1992; Strauss *et al.* 2002; Strauss & Zangerl 2002; Endara & Coley 2010). Although generalist herbivores are often deterred by secondary compounds, the components of secondary compounds can be detoxified and utilised either for growth or the herbivore's own defence against natural enemies, driving the evolution of specialist herbivores (Cates & Rhoades 1977; Cornell & Hawkins 2003).

1.2.1.1 Elemental Defence Hypothesis:

The uptake of very high concentrations of metal ions into plant shoots as another chemical defence strategy has been proposed as an alternative to organic defence compounds. The toxic effects of metals on animals have been well researched in their use as fungicides and insecticides and from environmental pollution (Alloway & Ayres 1997; Kabata-Pendias & Pendias 2001). 'Elemental Defence' as proposed by (Boyd & Martens 1992) is the uptake of metals into shoots at very high concentrations, thus potentially producing sub-lethal and lethal effects to herbivores and pathogens through deterrence

and fatality. It has three potential advantages over organic chemical defences, which are summarised below (Boyd & Martens 1992; Martens & Boyd 1994; Boyd 1998; Boyd 2007):

- 1) Metals, as elements, cannot be chemically degraded like their organic defence molecules counterparts. To detoxify metal-ions, especially at the extremely high concentrations found in hyperaccumulators, would require an herbivore to evolve the ability to complex and sequester metal-ions. Additionally, metal-toxicity could be diluted through the consumption of high quantities of low-metal foliage. Thus the use of metals as a defence strategy would be effective against generalist herbivores with a low-metal tolerance.
- 2) Although the tolerance of metal ions in the plant requires complexation by organic acids e.g. oxalate, malate and citrate, and by metallothioneins and phytochelatins (cysteine-rich metal-binding peptides) (Hall 2002), these complexes are relatively low-weight, and hence are seen as less 'costly' to the plant when compared to other high-weight organic defence compounds, such as cyanogenic glucosides and glucosinolates.
- 3) The use of metals in herbivore defence is seen as a novel strategy because of the restricted distribution of metalicolous communities and their elevated foliar metal concentrations. This would be an advantage when defending against generalist herbivores as they are unlikely to have evolved metal-resistance.

To use metals as an herbivore defence strategy, a sufficient concentration of bioavailable metal in the soil is required that the plant can access, tolerate and transport to its shoots (Boyd & Martens 1992). Thus the proposal of the Elemental Defence Hypothesis came from the observation of extremely high foliar metal concentrations in plants (hyperaccumulators) growing on metal-rich soils, growing alongside other plant species with relatively lower foliar

metal concentrations (Boyd & Martens 1992). This makes elemental defence an unusual defence strategy, as it is likely to be restricted to metal-tolerant plants growing on metal-rich soils (Boyd & Martens 1992).

1.3 Metalliferous Soils and their Plant Community:

1.3.1 *Metalliferous Soils:*

Metalliferous soils have elevated concentrations of metals from weathering of metal-rich rock or anthropogenic activities such as mining and smelting. As well as their elevated concentration of metals (some of which are toxic), metalliferous soils usually have a low nutrient status (especially in nitrogen and phosphates) and are free-draining and sometimes acidic, with the bioavailability of the metals present to the plants influenced by other abiotic factors, such as the pH and organic matter content (Smith & Bradshaw 1970; Antonovics, Bradshaw & Turner 1971; Gemmell 1977; Baker 1987). Although all plants require trace levels of a variety of inorganic mineral nutrients for optimal growth, development and reproduction; excessive concentrations of these metals in the soil can cause toxicity symptoms in non-adapted plants (Foy, Chaney & White 1978; Baker & Walker 1990; Marschner 1995; Hall 2002; Sridhar *et al.* 2005). Toxicity can result in inactivating enzymes, disrupting the chemical and physical structure of membranes, interfering with and blocking the functional groups of metabolites, and displacing or substituting for other chemically similar essential elements (Foy, Chaney & White 1978; Marschner 1995; Briat & Lebrun 1999; Kabata-Pendias & Pendias 2001; Hall 2002; Rascio & Navari-Izzo 2011). Thus, for plants to grow in metalliferous soils requires increased metal-resistance, either through avoidance of the metals or tolerance of their effects, frequently to multiple metals (Foy, Chaney & White 1978; Verkleij & Schat 1990; Briat & Lebrun 1999; Hall 2002). This has resulted in the selection for metal-resistance plant species and populations, resulting in a

unique type of plant community (Clark & Clark 1981; Baker 1987; Ernst 1990; Wu 1990; Macnair 1997).

1.3.2 *Metallicolous Plant Communities:*

Metallicolous plant communities occur on mineral veins and their outcrops and mine spoils. Metallicolous plant communities are unique because of the complex combination of stressful abiotic conditions such as metal toxicity, low nutrient status, free-draining and unstable soil structure, which makes conditions harsh and unsuitable for many common open grassland dominant plants (Smith & Bradshaw 1979), allowing rare plant and cryptogam species to flourish (Antonovics, Bradshaw & Turner 1971; Rodwell *et al.* 2007; Baker *et al.* 2010). Metalliferous soils are populated by a mixture of plant species and ecotypes which can tolerate the high metal concentrations, forming a distinctive plant community in the UK (Rodwell *et al.* 2007). Plant species (or sometimes subspecies and ecotypes) which are restricted to, and survive and reproduce on metalliferous soils are called metallophytes (Antonovics, Bradshaw & Turner 1971; Baker *et al.* 2010). Metal-tolerant genotypes found on metalliferous soils, result from the natural selection of individuals with a greater metal tolerance from the surrounding non-metallicolous populations, leading to the formation of metallicolous populations, and eventually speciation (Antonovics, Bradshaw & Turner 1971; Baker *et al.* 2010). Metallophytes can be divided into two groups – obligate (or absolute) metallophytes and facultative metallophytes. Obligate metallophytes are plant species restricted to metalliferous soils, and have a narrow ecological niche, e.g. *Alyssum pintodasilvae*, *Viola guestphalica* and *Viola lutea* subsp. *calaminaria* in Europe (Baker *et al.* 2010). Facultative metallophytes are plants species which are common to non-metalliferous phytogeographical regions but also form specific metal-tolerant genotypes or ecotype/ sub-species which are restricted to metalliferous soils, e.g. *Armeria maritima*, *Minuartia verna*,

Silene vulgaris and *Thlaspi caerulescens* in Europe (Baker *et al.* 2010).

Metallicolous plant communities are also composed of plant species whose distribution is not dependent on the presence of metalliferous soils. These species termed 'associate metal-tolerant species' have a wide geographic distribution and abundance but also have a moderate metal-tolerance capability and can tolerate nutrient-poor and unstable soils (Baker *et al.* 2010). 'Associate metal-tolerant species' mainly comprise 'pseudometallophytes', i.e. plant species which form tolerant populations on contaminated soils, and non-tolerant populations on non-contaminated soils, within the same geographical region (Baker *et al.* 2010). Separate gene pools may be maintained despite gene flow between spatially close pools, due to the selective pressure of metal toxicity on metalliferous soils and biotic competitive interference on non-metalliferous soils, as seen for *Agrostis tenuis* on a copper mine (McNeilly, 1968). Additional examples of 'pseudometallophytes' include *Achillea millefolium*, *Campanula rotundifolia*, *Euphrasia* spp. *Plantago lanceolata*, *Polygala vulgaris*, *Ranunculus acris*, *Rumex acetosella*, *Rumex acetosa*, *Thymus pulegioides*, *Agrostis capillaris*, *Festuca rubra*, *Festuca ovina*, *Holcus lanatus* and *Phragmites australis* (Antonovics, Bradshaw & Turner 1971; Baker & Proctor 1990; Baker *et al.* 2010).

1.4 Adaptations of Plants to Metal-Rich Soils:

1.4.1 *Metal Uptake in Non-Metalliferous Soils:*

'Normal', non-metalliferous soils have trace levels of metal ions in the soil, which are generally essential to plant growth. Thus in higher plants, transportation of essential metal elements from the soil into the root hair cells generally requires metabolic energy (called 'active transport') and the presence of membrane transporter proteins (Marschner 1995). Root exudates are used to free the metal ions from their bound form in the soil first (i.e. to make them phytoavailable), and regulatory mechanisms are used to ensure correct delivery

of the ions to their recipient cells. Metal ions that are not required are then eliminated, either by excretion or internally storing them in the cell vacuole (Marschner 1995). In soils where there is an adequate but not excessive supply of metals, higher plants generally accumulate and immobilize trace metals in their roots, and this metal 'exclusion' restricts the supply of metals upwards into the aerial parts (Baker & Walker 1990; Kabata-Pendias & Pendias 2001). The relationship between soil metal concentration and foliar metal concentration is usually linear in 'normal' soils (Kabata-Pendias & Pendias 2001), although this is metal specific (Baker & Walker 1990).

On metalliferous soils however, it seems that passive uptake of metals into the roots of metal-tolerant plants is very high, and higher plants cannot prevent excessive metal uptake, but can only restrict uptake into their roots and accumulate metals in their tissue to a varying amount (Peterson 1971; Baker 1981; Baker & Walker 1990). Thus in metalliferous soils, physiological tolerance mechanisms are required to restrict metal uptake in the roots, reduce root-to-shoot translocation, thus reducing the build-up of metals to toxic cellular concentrations, and to tolerate the presence of metals in the shoots (Briat & Lebrun 1999; Hall 2002).

1.4.2 *Metal Tolerance:*

The exposure to an excess of metals in the soil over thousands of years has selected for metal resistance (the ability to survive and reproduce in stressful conditions) (Antonovics, Bradshaw & Turner 1971; Baker 1987). Metal resistance usually takes the form of metal tolerance traits which use specific physiological mechanisms to cope with the effects of internal metal stress (Baker 1987). Metal tolerance is the most common strategy and involves a spectrum of biochemical and physiological adaptations (Baker 1987; Baker &

Walker 1990). Metal tolerance can refer both to 1) a species growing on metalliferous soil, whereby other plant species are excluded from growing there, hence processing a constitutional tolerance within the species; and 2) to physiologically-adapted individuals of a regionally common species forming a distinct metal-tolerant population (Antonovics, Bradshaw & Turner 1971; Kabata-Pendias & Pendias 2001). It seems that only species which possess a variability in their metal tolerance within normal populations, are able to evolve metal-tolerant populations that can colonised metalliferous soil (Baker 1981; Baker 1987). However this species must also be able to tolerate the harsh abiotic conditions found on metalliferous soils as well, such as their low nutrient status (Baker 1987).

1.4.2.2 Physiological Metal Tolerance Mechanisms:

A plant's ability to restrict and tolerate elevated soil metal concentrations is based on both extra-cellular and intra-cellular mechanisms. Extra-cellular mechanisms include the degree of metal mobility in the soil, the production of root exudates to bind metals to the cell wall, and the presence of ameliorating mycorrhizal interactions (Baker & Walker 1990; Marschner 1995; Kabata-Pendias & Pendias 2001; Hall 2002). There are several potential cellular physiological mechanisms within the plant (Baker & Walker 1990; Marschner 1995; Kabata-Pendias & Pendias 2001; Hall 2002). The majority of these intra-cellular metal tolerance mechanisms primarily avoid the build-up of metals to toxic concentrations at sensitive sites within the cell, rather than display enhanced stress-resistance (Briat & Lebrun 1999; Hall 2002). These include regulating the metal uptake into the cell through the plasma membrane (either by reduced influx or by activating the efflux), complexing the metals using phytochelatins, metallothioneins, organic acids and amino acids in the cell cytosol and vacuole to render them immobile, and restricted root-to shoot

translocation (Baker 1987; Marschner 1995; Briat & Lebrun 1999; Kabata-Pendias & Pendias 2001; Hall 2002). However, the specific mechanisms and their importance is dependent upon the plant species and genotype, and the metal type involved, and additionally may involve multiple mechanisms at the cellular level and various alterations and adaptations at the whole-plant level (Kabata-Pendias & Pendias 2001; Hall 2002).

1.4.2.3 Excluders, Indicators, Accumulators and Hyperaccumulators:

The metal tolerance mechanisms described above are typical 'exclusion' mechanisms whereby tolerance is achieved through restricting metal uptake in the roots, with minimum shoot translocation, and is a common strategy for metal-tolerant plants (Baker 1981; Baker & Walker 1990; Rascio & Navari-Izzo 2011). For instance, Baker (1978) showed that the response to a Zn-amended nutrient solution differed between coastal and mine populations of *Silene maritima*. Both populations were able to restrict the movement of Zn from the roots to the shoots up to a certain Zn nutrient-solution concentration, but when this threshold was reached, the mine population accumulated more Zn in the roots, whereas the coastal population accumulated more in the shoots. Hence excluders generally have a low shoot: root metal concentration ratio (the 'concentration factor') of <1. This restriction occurs until a critical soil metal concentration is reached and overrides this mechanisms resulting in unrestricted transport (Baker 1981; Baker & Walker 1990).

Baker (1981) defined another two metal tolerance strategies: 'Indicators' and 'Accumulators'. 'Indicators' regulate the shoot metal concentration so that a linear relationship between plant and soil metal concentrations are produced (Baker 1981). 'Accumulators' actively concentrate metals in their aerial parts, regardless of the of soil concentration, and produce shoot concentrations that

are higher than the roots (hence ratio of ≥ 1), although the roots will still contain an elevated concentrations (Baker 1981). Their physiological metal tolerance strategy may be linked to their geographical distribution, as metallophytes are often 'accumulators' and pseudometallophytes are often 'excluders' (Baker 1981; Baker & Walker 1990). Thus the shoot: root Zn concentrations of *Silene vulgaris* and *Armeria maritima* are under 1, displaying an exclusion strategy; whereas *Thlaspi* spp. and *Minuartia verna* have shoot: root Zn concentration ratios of >1 , displaying the accumulation strategy (Baker 1981). However this is dependent upon the metal type and its soil metal concentration as well as the population studied (Baker 1981). For instance, Ernst (1975) looked at 12 plant species growing on a naturally highly metalliferous soil in Germany. *Thlaspi alpestre* (synonym *Thlaspi caerulescens*), *Minuartia verna* and *Armeria maritima* displayed much higher shoot concentrations of Zn, lead (Pb), cadmium (Cd) and copper (Cu), than the other species including *Silene vulgaris*, *Festuca ovina* and *Rumex acetosa*. However, all species displayed significantly high foliar metal concentrations that would be toxic to non-tolerant populations. The degree of uptake, transportation and accumulation is dependent on the type of element present, the plant species or population, and possibly the physiological distinction between essential and non-essential elements (Baker 1981; Baker & Walker 1990).

Plants that have extremely high foliar metal concentrations in relation to their root values are termed 'hyperaccumulators'. This phrase was first used by Brooks *et al.* (1977) to describe the foliar nickel (Ni) concentrations seen in a selection of plant herbarium species collected from ultrabasic soil (Ni and chromium (Cr) enriched). The foliar Ni concentration of plants found on normal soils is around 15 $\mu\text{g/g}$, and usually $< 100 \mu\text{g/g}$ on ultrabasic soils. However Brooks *et al.* (1977) found several species (mainly from the genera *Homalium* and *Hybanthus*) with highly elevated foliar Ni concentrations and classed them as

strong accumulators (100 - 1000 µg/g) and hyperaccumulators (> 1000 µg/g). Further investigation of other elements revealed extraordinarily high foliar concentrations in selected metalicolous plants species and the designation of hyperaccumulator threshold concentrations. For instance, Zn hyperaccumulation is defined as 10,000 µg/g because of the presence of certain plants species growing in the Zn-rich soils of central Europe (mainly *Thlaspi* spp.) with foliar Zn concentrations equal or above this – tenfold greater than other metalicolous plant species (Baker & Brooks 1989). Hence hyperaccumulators show the ‘accumulation’ strategy (Baker 1981), i.e. a greater metal uptake ability from the soil, an increased root to shoot metal translocation ability and are to detoxify and sequester greater quantities of metal in the shoots (Baker 1981; Macnair 2003; Yang *et al.* 2005; Verbruggen, Hermans & Schat 2008; Rascio & Navari-Izzo 2011). The physiological mechanisms underpinning this phenomenon have been mainly explored using the hyperaccumulators *T. caerulescens* and *Arabidopsis halleri* (Lasat & Kochian 2000; Pence *et al.* 2000; Assunção, Schat & Aarts 2003; Milner & Kochian 2008).

The selective advantage of hyperaccumulation still remains unclear, especially as many metalicolous species and populations do not display this unique strategy, and because of extensive within-population variation in hyperaccumulators (Macnair 2003; Zhao, Lombi & McGrath 2003; Rascio & Navari-Izzo 2011). Nevertheless, the evolution of the hyperaccumulation trait across many clades, the potential cost of shoot detoxification and tolerance (Maestri *et al.* 2010), and the hyperaccumulation (Morrison, Brooks & Reeves 1980) and strong accumulation of metals (Reeves & Baker 1984; Bert *et al.* 2002; Pongrac *et al.* 2009) even when grown on low-metal substrate soils, suggests an adaptive advantage (Boyd & Martens 1992; Macnair 2003).

1.5 Ecological Advantages of Hyperaccumulation:

Boyd & Martens (1992) initially proposed five possible ecological advantages for metal hyperaccumulation, which are summarised below (Boyd & Martens 1992; Boyd 1998; Boyd & Martens 1998b; Macnair 2003; Rascio & Navari-Izzo 2011):

1. **Tolerance/ Disposal Hypothesis:** Hyperaccumulation may allow tolerance to excessive soil concentrations by transporting and complexing metals in shoot. However this requires specialist physiological mechanisms and potentially incurs a 'cost'. High concentrations of metal in the aerial plant parts may allow possible metal disposal through leaf shedding and leaching from rainfall. However, the majority of metallicolous plants display an 'excluder' strategy, and the relationship between metal-tolerance and accumulation is unclear. The ecological consequence of increased metal-concentrations in leaf litter is unknown.
2. **Drought Resistance Hypothesis:** Metalliferous soils frequently have a low water and nutrient status. This hypothesis was suggested as metals are stored in leaf epidermal cells and because metal-elements influence biochemical responses to water stress. However, very little research has yet been done to show if metals relieve water-stress more so than other known organic compounds. Additionally, not all metalliferous soils have very high water stress, so this hypothesis may not be a universal explanation for accumulation.
3. **Interference Hypothesis:** The deposition of metals in the leaf litter surrounding a hyperaccumulating plant may be a competitive strategy, to prevent the growth of metal-sensitive plants around the immediate vicinity of the hyperaccumulator. This hypothesis is again little researched but potentially not supported as hyperaccumulator leaf litter did not inhibit seed germination of competing plants. Additionally, although hyperaccumulators have been

observed growing in pure stands, this strategy requires the hyperaccumulators to be more metal-tolerant than their competitors. Although interference may be an advantage it is probably not the main selective advantage of metal tolerance and hyperaccumulation.

4. Inadvertent Uptake Hypothesis: This hypothesis suggests that enhanced shoot translocation of metals is a result of other physiological processes such as increased water transpiration rate or nutrient acquisition, and is a non-adaptive feature. It is supported by the hyperaccumulators' characteristic features of highly efficient metal uptake mechanism, multiple-metal hyperaccumulation and ability of closely related non-metallicolous species to accumulate metals. Again, this hypothesis has been little studied and thus there is lack of evidence for nutrient deficiencies in hyperaccumulators.

5. Elemental Defence Hypothesis (EDH): High concentrations of some metals are toxic to both animals and microbes (e.g. their use in fungicides and insecticides), and interfere with their biochemical pathways in a multitude of ways. The EDH was initially proposed as the extremely high foliar metal concentrations of hyperaccumulators produce lethal effects in invertebrate herbivores and reduced fungal attack.

All of these hypotheses may be relevant to some habitats and plant species and are probably not mutually exclusive (Boyd 1998), but the most widely applicable and well-researched of them is the EDH (Boyd 2004). Recent research has provided much support for the extremely high metal concentrations in hyperaccumulating plants to deter and produce fatal effects in both herbivores and plant pathogens (Poschenrieder, Tolrà & Barceló 2006; Boyd 2007; Vesik & Reichman 2009). Laboratory studies using Ni, Cd, selenium (Se) and Zn hyperaccumulating plants have shown negative effects of metal hyperaccumulation on generalist herbivores (Pollard & Baker 1997; Boyd *et al.* 2002; Jiang *et al.* 2005; Freeman *et al.* 2007) and plant pathogens (Freeman *et al.*

2005; Poschenrieder, Tolrà & Barceló 2006), and to a lesser extent in the field with generalist invertebrate herbivores (Martens & Boyd 2002; Noret *et al.* 2007b; Galeas *et al.* 2008). However, defence is not always conferred with high foliar metal concentrations, and can be dependent upon herbivore feeding mode (Jhee, Boyd & Eubanks 2005) or specialist metal-tolerant herbivores taking advantage of high foliar metal concentrations for their own defence against natural enemies (Boyd & Wall 2001; Freeman *et al.* 2006).

The ability of lower concentrations of metals than those occurring in hyper-accumulating plants to deter herbivory has been very little researched, nor has the interaction of metal concentrations with other aspects of plant palatability (such as organic defences) been investigated. This is an important knowledge gap and studies which address it have the potential to extend the EDH to non-hyperaccumulating plants (Boyd 1998; Hanson *et al.* 2003; Coleman, Boyd & Eubanks 2005; Jhee, Boyd & Eubanks 2006; Boyd 2007).

1.5.1 *The Effect of 'Accumulator' Levels of Metals on Herbivores:*

Research testing the EDH has often overlooked the defensive effects of foliar metal concentration below hyperaccumulator levels (Boyd 2007), despite the fact that the relatively lower foliar metal concentrations of metal-tolerant and non-hyperaccumulating plants could also make them less palatable to herbivores (Boyd & Moar 1999). The benefits of herbivore deterrence from the higher foliar metal concentration of an individual plant, compared to the surrounding vegetation, may have produced a step-wise evolutionary pathway to increased metal tolerance, foliar accumulation and hence hyperaccumulation (Boyd 1998; Boyd 2004). The reduced palatability from the elevated foliar metal concentrations of metallicolous non-hyperaccumulating species has been demonstrated in a few studies, mainly for Se and Ni (Brooks 1987; Boyd & Moar

1999; Hanson *et al.* 2003), although one case has been documented for reduced Lepidoptera larvae performance on the Zn accumulator *Viola calaminaria* (Noret *et al.* 2007a). Globally there are around 10-20 Zn hyperaccumulating plant species, but there are about 40 plant species that can 'accumulate' at least 3000 mg/kg Zn in their shoots on Zn-rich soils (Reeves & Baker 2000). Thus, further research is required to investigate the influence of 'defensive enhancement' using 'accumulator' concentrations on plant palatability (Boyd 2007), which this thesis has undertaken using the elevated foliar Zn concentrations found in metal-tolerant populations of the pseudometallophyte *Rumex acetosa*.

1.6 Potential Costs to Metal Tolerance and Accumulation:

1.6.1 *Competitive Ability:*

Metallicolous plant populations are subjected to a harsh abiotic environment, which often has a low water and nutrient supply in addition to the high concentrations of toxic metals (Antonovics, Bradshaw & Turner 1971; Baker 1987; Ye *et al.* 2002). The physiological adaptations to these conditions potentially requires additional energy, resulting in a metabolic cost to the plant (Wilson 1988; Punz & Sieghardt 1993; Ernst 2006; Maestri *et al.* 2010). These metabolic costs may result in survival in metal-contaminated soil at the expense of biomass and reproductive output, i.e. there may be a trade-off between metal tolerance and fitness (Baker 1987; Ernst, Schat & Verkleij 1990; Wu 1990; Harper, Smith & Macnair 1997a; Kazakou *et al.* 2008; Maestri *et al.* 2010). Superior metal tolerance and growth on mining sites is therefore potentially dependent upon the ability of an individual to capture resources in low nutrient-status soils. The relationship between metal tolerance and competitive ability has been investigated between non-metallicolous and metal-tolerant plant species and populations and has shown that the metal tolerance trait has a possible productivity cost associated to it, thus rendering metal-tolerant plants

competitively inferior to their non-metal-tolerant counterparts (Antonovics, Bradshaw & Turner 1971; Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975; Wilson 1988). In addition, the greater density of neighbouring plants typical of non-metallicolous communities compared with typical metallicolous ones means plants from non-metallicolous communities are potentially more adapted to, and tolerant of, plant competition for nutrients (Keddy 1989; Grime 2002). However, apparent trade-offs between metal tolerance and competitive ability have to be interpreted carefully as other traits which differ between metallicolous and non-metallicolous populations may contribute to the population differences seen (Higgins & Mack 1987; Wilson 1988; Ernst 2006). An alternative approach is to examine the competitive abilities of different metallicolous populations derived from locations which vary in their soil metal concentrations and vegetation cover. This thesis also investigates how the competitive abilities of such contrasting metallicolous populations are influenced by both the soil metal concentration and the presence of generalist herbivores.

1.7 Study System:

This section describes the metalliferous region, metal, plant species and the generalist herbivore used in this thesis and explains why they were chosen as a study system.

1.7.1 *The Pennines:*

Due to their natural geology, the Pennines in northern England are major ore fields of Zn, Pb and Cd and have been mined for the past 2000 years, leaving behind metal-rich spoils colonised by a unique metal-tolerant plant community (Shimwell & Laurie 1972; Ford & Rieuwerts 1975; Morrey, Baker & Cooke 1988; Baker & Proctor 1990). Waste rock left on the surface in spoils and tailings has

eroded to produce Zn and Pb-rich soils on the limestone beds, and the associated vegetation called calamine grasslands, has evolved to tolerate the highly toxic and nutrient-poor soils (Rodwell *et al.* 2007). Soil Zn and Pb concentrations of 42,000 and 76,500 mg/kg respectively have been recorded (Morrey, Baker & Cooke 1988), approximately 100 times that of non-contaminated soils (Broadley *et al.* 2007). Thus the metallicolous plant communities within the Pennines provide a suitable study system for which to investigate the influence of Zn accumulation on plant-herbivore interactions.

1.7.2 Zinc:

Zn is an essential element for plants and is generally present in a range of mineralogical forms at trace concentrations in the soil (Marschner 1995), typically around 10 - 300 µg/g total Zn (Lindsay 1972; Kiekens 1995; Broadley *et al.* 2007). At these concentrations, Zn is available to plants either as soluble Zn²⁺ ions from the soil solution, soluble zinc-organic complexes through binding with fulvic acids, and from the production of root exudates such as organic acids (e.g. citrate and oxalate) and amino acids to chelate metal ions (Lindsay 1972). Plants take up available Zn usually through active transport, or as an organic complex, from the low concentration soil solution, and using Zn transporters move it into their roots, where it is then transported to the aerial parts through the xylem (Lindsay 1972; Marschner 1995; Kabata-Pendias & Pendias 2001; Broadley *et al.* 2007). There is usually a linear relationship between the soil and foliar Zn concentrations under non-metalliferous growth conditions (Antonovics, Bradshaw & Turner 1971; Barry & Clark 1978; Kabata-Pendias & Pendias 2001).

In the shoots, Zn plays an important role in the plant's biochemistry, where it is involved in carbohydrate metabolism, protein synthesis, cellular membrane

structure, pollen formation and as a regulator in auxin synthesis (Lindsay 1972; Kiekens 1995; Marschner 1995; Broadley *et al.* 2007; Alloway 2009). Zinc is also used to regulate and maintain the genes involved in abiotic stress tolerance, such as high temperatures and light intensity (Kiekens 1995; Alloway 2009) and biotic stress (Kabata-Pendias & Pendias 2001; Poschenrieder, Tolrà & Barceló 2006). A typical leaf Zn concentrations is 15 - 20 mg/ kg DW (Marschner 1995) although mature leaves can be 25 - 150 mg/kg (Kiekens 1995). The internal transport of Zn within a plant can vary, but Zn is generally highest in the roots, stored in the root cell vacuoles (Kabata-Pendias & Pendias 2001), and also concentrated in mature leaves (Antonovics, Bradshaw & Turner 1971).

Soil Zn concentrations higher than 300 µg/g total Zn are generally regarded as phytotoxic (Kiekens 1995; Kabata-Pendias & Pendias 2001), as plants generally cannot prevent excessive movement of Zn ions through diffusion (passive uptake) into their roots and shoots (Lindsay 1972; Marschner 1995). Leaf Zn concentrations are regarded as 'excessive' or 'toxic' if greater than 400 mg/kg (Kiekens 1995), although toxicity thresholds can vary within genera and within species (Kabata-Pendias & Pendias 2001; Broadley *et al.* 2007). Zn toxicity symptoms include reduced shoot and root biomass and growth rate, and affects iron, phosphorous and magnesium uptake (Marschner 1995; Hall 2002; Broadley *et al.* 2007) and altered protein structures (Marschner 1995; Hall 2002). Zn-tolerant plant species and genotypes however can negate the toxic effects by metabolic adaptation and metal-complexing, or by restricting uptake into the plant and into cells, or by immobilizing the metal in storage tissues (Kabata-Pendias & Pendias 2001). The fact that Zn is essential for plants, but has toxic effects at high soil and foliar concentrations, make it a suitable metal to study when testing the EDH.

1.7.3 *Thlaspi caerulescens*:

Much of the research on metal-tolerance and hyperaccumulation has focused upon the Zn and Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Pres.) (Alpine Pennycress) (synonym *Noccaea caerulescens* (J. & C. Presl.) F. K. Meyer) (Brassicaceae) e.g. (Baker & Brooks 1989; Boyd & Martens 1998a; Lombi *et al.* 2000; Assunção, Schat & Aarts 2003; Zhao, Lombi & McGrath 2003). *T. caerulescens* is classed as a facultative metallophyte (Baker *et al.* 2010) as it has been found to grow on a wide range of soil Zn concentrations in the field and greenhouse – from approximately 50 to 50,000 mg/kg (Zhao, Lombi & McGrath 2003), concentrations typical of normal soil to those of naturally mineralised rock and metal-mining waste sites (Broadley *et al.* 2007). It accumulates Zn in its aerial parts in a log-linear fashion, (ranging from 500 to 70,000 mg/kg) thus hyperaccumulating Zn on the basis of the soil to shoot concentration ratio (Zhao, Lombi & McGrath 2003), and the 10,000 mg/kg threshold limit when grown under Zn-enriched soils (Baker & Brooks 1989). British metallicolous populations of *T. caerulescens* are tolerant to a variety of metals (Baker, Reeves & Hajar 1994). In England and Wales, *T. caerulescens* is only found on metalliferous soils with moderate to high concentrations of extractable Zn or Pb (Ingrouille & Smirnov 1986). Thus its association with mining sites and naturally mineralised soils seems to restrict its distribution to the major metal-ore areas of the Pennines, the Mendips, and North and Central Wales (Baker, Reeves & Hajar 1994) and to abandoned mines on non-calcareous soils in North Yorkshire (García-Gonzalez & Clark 1989).

The geographical population variation in British *T. caerulescens* morphology, metal tolerance and ability to accumulate metals, such as Zn, Pb and Cd, indicates that there is genetic variation acted upon by natural selection (Ingrouille & Smirnov 1986; Pollard & Baker 1996). Field studies in continental

Europe have shown differences between these metallicolous and non-metallicolous populations in their growth and tolerance to metal-contaminated soils (Escarré *et al.* 2000), Cd and Zn tolerance and concentrations (Escarré *et al.* 2000; Roosens *et al.* 2003), and life history traits (Dechamps *et al.* 2007; Dechamps *et al.* 2008; Dechamps *et al.* 2011). This suggests the potential for local adaptation to abiotic and biotic site conditions (Jiménez-Ambriz *et al.* 2007; Dechamps *et al.* 2008).

Out of the eight studies investigating the effectiveness of Zn as an elemental defence, six have used *T. caerulescens*. However, conflicting results have been found in terms of the impacts on generalist, chewing herbivores (Boyd 2007). For instance, Pollard & Baker (1997) and Behmer *et al.* (2005) grew *T. caerulescens* in low and high growth medium Zn treatments, and found that the higher foliar Zn concentration produced by the growth medium concentration negatively influenced plant palatability and herbivore performance. Jhee *et al.* (1999) used genetic variation in *T. caerulescens* foliar Zn concentration and found the avoidance of high Zn leaves by late-instar caterpillars but not early-instar caterpillars. However no influence of foliar Zn concentration was found on the damage by leaf-scraping thrips (Jiang *et al.* 2005). Additionally, the influence of Zn was found to be less important than the influence of organic defences such as glucosinolates, when comparing metallicolous and non-metallicolous populations in the laboratory (Noret *et al.* 2005) and field (Noret *et al.* 2007b). Thus, the role of elemental defence remains unclear even in a relatively well-studied hyperaccumulator species, so understanding the factors influencing its effectiveness requires further research.

1.7.4 *Rumex acetosa*:

Rumex acetosa (L.) (Common Sorrel) (Polygonaceae) is common throughout the British Isles and is mainly found in neutral or basic grassland, although it can be found on a variety of soil types at different altitudes (Lousley & Kent 1981). Like other members of the Polygonaceae, it is a fast growing perennial, with a long seed viability and is able to survive in disturbed land (Lousley & Kent 1981; Salt & Whittaker 1998). *R. acetosa* frequently occurs on heavy metal spoil (Salt & Whittaker 1998; Rodwell *et al.* 2007) and has elevated levels of Zn in its shoots and roots when grown in contaminated soils (Brown 1994; Wang *et al.* 2003; Barrutia *et al.* 2009; Epelde *et al.* 2010). Due to its distribution it is classed as a pseudometallophyte (Antonovics, Bradshaw & Turner 1971; Barrutia *et al.* 2009). As shoot and root concentrations are similar for plants growing metalliferous environments, as shown for a variety of metals by Wang *et al.* (2003), it is generally classed as an indicator due to its metal tolerance strategy, although high soil concentrations can produce a metal-exclusion strategy (Epelde *et al.* 2010). In the Pennines, Shimwell & Laurie (1972) reported foliar Zn concentrations of 600 to 3400 mg/kg when found growing in soils ranging from 800 to 35,000 mg/kg. Johnston and Proctor (1977) similarly found a foliar Zn concentration of 5000 mg/kg when growing in ~2500 mg/kg soil Zn concentrations, although a foliar Zn concentration of 11,000 mg/kg was reported in one specimen. Although *R. acetosa* does not 'accumulate' metal as according to Baker's physiological metal-tolerance strategies (1981), these foliar Zn concentrations are unusually high compared to other metal-tolerant plants *in situ* (generally 50-500 mg/kg in Zn-rich soils) and therefore the foliar Zn concentration of *R. acetosa* can be classified in the 'accumulator' category according to Reeves & Baker (2000). Nevertheless, although *R. acetosa* frequently occurs on mining sites where it has elevated foliar Zn concentrations, it has not been used in studies of the EDH before. These elevated foliar Zn concentrations

are comparable to those which have been shown to have adverse impacts on herbivores in previous studies using other accumulator and hyperaccumulator species, so this species is an interesting candidate to test the EDH. It has a wider distribution and ecological niche than hyperaccumulators from the same mining sites and also does not rely exclusively on elemental defence: the Polygonaceae family also contain high quantities of calcium oxalate, a potential organic chemical defence (Salt & Whittaker 1998; Nakata 2003). Potential herbivores of *R. acetosa* and the Polygonaceae are specialists such as leaf-mining fly larvae (e.g. *Pegomya* spp.), chrysomelid beetles and weevils from the *Apion* spp. (Salt & Whittaker 1998). Generalists are also important herbivores, such as larvae of Lepidoptera and Coleoptera, and also the Orthoptera and Mollusca (Salt & Whittaker 1998; Strauss & Zangerl 2002).

1.7.5 *Helix aspersa*:

Helix aspersa (Müller) (Gastropoda) (synonym *Cornu aspersum*), is a generalist herbivore and is widespread in Britain, throughout many habitats (Kerney & Cameron 1979). Despite its broad diet, it does display food preferences – in the field Iglesias & Castillejo (1999) found that *H. aspersa* displayed a strong preference for *Urtica dioica* possibly due to its higher protein and calcium content and Chevalier, Le Coz-Bouhnik & Charrier (2003) found that plant species with a high calcium and a lower Zn concentration were eaten more. *H. aspersa* has been frequently used in ecotoxicology studies on the effects of metals such as Zn, Pb, Cd and Cu on the growth and metal uptake of snail populations from metalliferous and non-metalliferous sites e.g. (Coughtrey & Martin 1977; Laskowski & Hopkin 1996b; Gomot-De Vaufléury 2000; Gomot-de Vaufléury & Kerhoas 2000; Beeby, Richmond & Herpé 2002). A few laboratory studies investigating the EDH have used *H. aspersa* as a generalist herbivore to test the impact of different metals on preference: Boyd *et al.* (2002) for Ni,

Huitson & Macnair (2003) for Zn, and Noret *et al.* (2005) for Zn and Cd.

Whereas a defensive function of Ni hyperaccumulation (compared to a non-hyperaccumulating plant species) was found against *H. aspersa* herbivory in Boyd *et al.* (2002), the effect of Zn and Cd on plant palatability was less clear as it was also influenced by the glucosinolate concentration (an organic chemical defence compound) of *T. caerulescens*, and by the growth medium Zn concentration in *Arabidopsis* crosses (Huitson & Macnair 2003). Thus the influence of metals on the feeding behaviour of *H. aspersa* remains unclear and requires further attention.

1.8 Outline of Thesis and Chapter Aims:

1.8.1 *Thesis Outline:*

This thesis investigates the Elemental Defence Hypothesis (EDH) using the Zn accumulator *Rumex acetosa* in both the field and in a series of greenhouse experiments. The EDH was proposed as a potential selective advantage of hyperaccumulation (Boyd & Martens 1992) and has been mainly investigated using hyperaccumulating plant species, which have generally confirmed this advantage (Boyd 2007; Vesik & Reichman 2009). Although there is the potential for the lower levels of metals found in accumulating plants to confer an herbivore defence, termed 'defensive enhancement', this is relatively less well studied (Boyd 2007). By comparing metallicolous populations from the Southern Pennines in the field and greenhouse, this thesis explores how potential population differences affect the response of the Zn accumulator *R. acetosa* to growth in elevated Zn concentrations, and how these different responses affect its palatability and competitive ability. This will allow us to gain a deeper understanding into how plants respond to metal-contaminated soils, and also how this affects plant-herbivore interactions and the competitive ability of plants to gain resources for metal tolerance.

1.8.2 Chapter Aims:

Chapter 2 presents a field study investigating the response to growth in Zn-rich soils of the hyperaccumulator *T. caerulescens* and the accumulator *R. acetosa*, for three abandoned Pb and Zn mining sites in the Southern Pennines. Relatively few studies have investigated the EDH in the field i.e. Martens & Boyd (2002), Noret *et al.* (2007b), Galeas *et al.* (2008) and Freeman *et al.* (2009), and, as yet, none have fully supported the EDH, potentially because of a failure to measure other plant and soil factors which may influence the growth of the plants and their vulnerability to herbivory. By measuring a range of soil parameters, such as the soil Zn concentration, pH and organic matter content, and plant parameters, such as the foliar Zn concentration, foliar damage levels and shoot biomass, and assessing the relationships between these parameters, this chapter aims to comprehensively assess the EDH in both *T. caerulescens* and *R. acetosa* under field conditions. Unusually, this study evaluates the EDH in the field in both different species and different populations of the same species. By comparing the relationship between the foliar Zn concentration and herbivory in a hyperaccumulating species and an accumulating one across three sites which differ in soil conditions, this chapter capitalises on between species differences in shoot uptake efficiency and on variation in foliar Zn concentration and herbivory between sites to test the EDH effectively.

Chapter 3 comprises two pot trials investigating the response of metalicolous *R. acetosa* populations to growth under moderately and highly contaminated levels of soil Zn and their palatability to the generalist snail *H. aspersa*. The aim of this chapter was to determine if there were population differences in foliar Zn concentration and, if so, to test whether these differences were sufficient to affect the palatability of the plants to adult and juvenile *H. aspersa*. Firstly, the growth of plants from seed (collected from the sites described in **Chapter 2**) and

their palatability was assessed under controlled conditions, confirming population differences between two of the three populations. In the second pot trial, the EDH was explored using a novel approach. Most studies investigate the EDH by altering the foliar metal concentration of the plants through manipulation of the growth medium's metal concentration. However growth under stressful elevated growth medium metal concentrations is likely to elicit other changes in the plant aside from the foliar metal concentration (Huitson & Macnair 2003), such as general physiological and biochemical effects from iron and phosphorus inhibition, chlorosis and stunted growth (Foy, Chaney & White 1978; Sridhar *et al.* 2005), organic defence compounds (Tolrà *et al.* 2001) and possible impairment of the stomatal function of *R. acetosa* (Barrutia *et al.* 2010). Thus, this approach does not allow a distinction between the potential changes in plant palatability due to the increased foliar metal concentration, and the potential changes in palatability from the physiological response to growth under elevated metal concentrations (Martens & Boyd 1994; Schwartz *et al.* 1999; Tolrà *et al.* 2001; Huitson & Macnair 2003; Tolrà *et al.* 2005; Boyd 2007). However, the use of genetic variation in foliar Zn concentration allows the influence of soil metal concentration on plant palatability to be controlled for (Jhee *et al.* 1999; Huitson & Macnair 2003). By exploiting metallicolous population differences in the foliar Zn concentration and also manipulating the foliar Zn concentration directly through the soil Zn concentration, this chapter aims to assess if plant palatability to adult and juvenile *H. aspersa* is influenced by the foliar Zn concentration *per se* or also by the plants' response to growth in moderately and highly contaminated soils.

Chapter 4 aims to investigate the potential cost to metal tolerance by measuring the competitive ability of individuals to capture resources for growth when growing in metal-rich soils. Studies investigating Zn and Pb tolerance have generally compared metallicolous with non-metallicolous populations, and due

to the reduction in biomass of the tolerant populations when grown under inter-population competition on normal soils, concluded that the metal-tolerance trait has a possible productivity cost associated to it (Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975; Wilson 1988). However, other adaptations to the conditions found at these two highly contrasting habitats may also influence the competitive ability of the respective populations, such as the potentially higher nutrient status of non-metalliferous soils and the influence of plant neighbours on the availability of resources (Higgins & Mack 1987; Wilson 1988; Keddy 1989; Grime 2002; Ernst 2006). By comparing two metalicolous populations derived from sites with elevated soil Zn concentrations, but with differences in their shoot Zn uptake rates, the potential costs of metal tolerance in terms of competitive ability can be measured without the confounding effects generated by comparing highly contrasting metalicolous and non-metallicolous populations derived from sites with extreme differences in soil Zn levels. This chapter investigates the impact of intra- and inter-population pairwise competition on the shoot biomass, relative competitive intensity (RCI), and foliar Zn concentration of two metal-tolerant populations of *R. acetosa*, and tests how these impacts differ between moderately and highly contaminated soil Zn concentrations.

Chapter 5 brings together the subjects of the two previous chapters by investigating how herbivory may affect the competitive ability of two metalicolous *R. acetosa* populations; it also assesses whether any impact of herbivory on competitive ability is altered by soil Zn concentration. Herbivory may shift the competitive balance between populations if the herbivores feed preferentially on one population at the expense of another (Crawley 1997). Which population is the most palatable may also be dependent on the soil Zn concentration. Using metalicolous populations of *R. acetosa* grown under inter-population competition, the effect of herbivory on the shoot biomass and foliar

Zn concentration was measured when the plants were grown in moderately and in highly contaminated soil Zn concentrations.

Finally **Chapter 6** summarises the key findings from this thesis and places them in the broader context of the EDH (Boyd 2007), plant-herbivore interactions, and plant competition. The use of 'accumulator' levels of foliar metal concentrations to deter herbivory as a potential 'defensive enhancement' mechanism is discussed with reference to a recent review by Boyd (2007), as is the potential interaction between elemental and organic defences. This chapter summarizes the important influence of Zn accumulation on both plant-herbivore interactions and on plant-plant interactions and highlights several areas which require further research to fully understand these interactions.

Chapter 2: Field Survey of the Shoot Biomass, Foliar Zinc Concentration and Herbivore Damage in *Thlaspi caerulescens* and *Rumex acetosa* at three abandoned lead/ zinc mines, Derbyshire.

2.1 Introduction:

Metallicolous plant communities are influenced by the severe abiotic environmental conditions present, chiefly their extremely high soil metal concentrations, thus driving the selection for metal tolerance characteristics (Baker 1987). Their elevated shoot metal concentrations are also thought to influence biotic interactions due to increased toxicity (Boyd & Martens 1998b). The Elemental Defence Hypothesis (EDH), first suggested by Boyd and Martens (1992) suggests that a higher shoot metal concentration protects the plant from herbivory due to deterrence and reduced herbivore performance. As hyperaccumulators have shoot metal concentrations typically 10-100 fold greater than other metallicolous plant species and populations (Brooks *et al.* 1977), this potential herbivore defence advantage could select for the extremely high shoot metal concentrations seen in hyperaccumulators (Boyd & Martens 1992; Boyd 2004). Although herbivore defence may not be the sole selective advantage to metal hyperaccumulation, it is one of the most researched, yet the EDH has been rarely tested in the field (Boyd 2007).

Although lab-based trials have generally demonstrated the deterrence of herbivory by hyperaccumulator concentrations of metals in plants (Boyd 2007; Vesik & Reichman 2009), no published field surveys of the impact of herbivores on UK metallicolous plant communities have been conducted. Field assessments of the degree of herbivore plant damage in metalliferous sites in light of the EDH are limited to Ni in California (Martens & Boyd 2002), Se in

Colorado (Freeman *et al.* 2009) and Zn in central Europe (Noret *et al.* 2007b). Additionally, Galeas *et al.* (2008) recorded the arthropod load in Se hyperaccumulators and non-hyperaccumulators in Colorado. Both the Se studies supported the EDH, in that plants with a higher Se concentration had a lower arthropod load (Galeas *et al.* 2008) and were protected from mammalian herbivory (Freeman *et al.* 2009). However neither the Ni nor Zn studies fully supported the EDH, potentially due to other confounding factors such as the influence of other plant defences on herbivore choice. Therefore the EDH still remains to be extensively tested under field conditions to conclusively support or reject this hypothesis. The field study reported in this chapter examines the potential relationship between the foliar Zn concentration and leaf tissue damage in two metallophytes, *Rumex acetosa* and *Thlaspi caerulescens*, which are known to contrast in their foliar Zn concentration due to differences in their shoot Zn uptake. As the foliar Zn concentration is dependant largely on the soil Zn concentration, the influence of abiotic site differences on the foliar Zn concentration and the ratio between soil and foliar Zn concentration (the concentration factor), also needs to be considered (Baker 1981).

Because of the elevated soil metal concentration, the most common mechanism to avoid metal toxicity in the plant, is by excluding root metal uptake and restricting root-to-shoot metal transport (Briat & Lebrun 1999). However because of the high soil metal concentrations, complete exclusion and restriction cannot occur, resulting in elevated foliar metal concentrations (Baker 1981; Baker & Walker 1990). Plant species differences in metal uptake have been recorded, which are also metal-specific (Baker 1981; Sieghardt 1990). Metallicolous populations of *R. acetosa* grown in contaminated soils demonstrate restricted Zn shoot transport compared to non-metallicolous populations (Baker & Walker 1990). Nevertheless, metallicolous populations of *R. acetosa* still exhibit elevated shoot metal concentrations in UK mining sites

(Hickey & McNeilly 1975; Thompson & Proctor 1983; Salt & Whittaker 1998) – foliar Zn concentrations of 600 - 3400 mg/kg (Shimwell & Laurie 1972) and 5000 mg/kg (Johnston & Proctor 1977) have been recorded for the Southern Pennines. As not all metal-tolerant plants display such elevated foliar Zn concentrations when grown *in situ*, generally reaching foliar Zn concentrations of 50-500 mg/kg, *R. acetosa* elevated foliar Zn concentrations are to be noted. Foliar Zn concentrations greater than 3000 mg/kg in Zn-rich soils are considered unusual, and therefore *R. acetosa* foliar Zn concentration can be classified in the ‘accumulator’ category according to Reeves & Baker (2000).

Elevated foliar metal concentrations interfere with many plant physiological shoot processes (Foy, Chaney & White 1978; Briat & Lebrun 1999), even in metal-tolerant plants (Sridhar *et al.* 2005). However, a group of plants with extremely elevated foliar metal concentrations, termed hyperaccumulators, demonstrate an active shoot uptake of metals, and increased soil- and shoot-metal tolerance (Rascio & Navari-Izzo 2011). The hyperaccumulation of Zn (i.e. ability to accumulate foliar concentrations exceeding 10,000 mg/kg, when grown in moderate and highly metal enriched soils) has been well documented in British populations of *T. caerulescens* (Shimwell & Laurie 1972; Baker, Reeves & Hajar 1994). The selective advantage of the hyperaccumulation trait is still unclear, and could be due to a variety of abiotic and biotic pressures (Boyd & Martens 1992). Most ecological research has investigated the potential advantage of herbivore deterrence offered by metal hyperaccumulation, but studies generally contrast metalliferous with non-metalliferous sites in the field, or compare elevated and normal shoot metal concentrations in lab feeding trials (Boyd & Moar 1999; Huitson & Macnair 2003). However these are not ecologically realistic representations of the potential selective advantages within a field site.

In this chapter, by assessing different metalliferous sites and comparing plant species with elevated foliar Zn concentrations, potential site differences will allow investigation into how the foliar Zn concentration, concentration factor and herbivore shoot damages varies between- and within-sites. The potential relationship between foliar Zn concentration and herbivory can be investigated using species contrasting in their foliar Zn concentration and metal tolerance strategies. Assessment of the foliar Zn concentration and shoot damage to the hyperaccumulator *T. caerulescens* and the accumulator *R. acetosa* will enable the EDH to be tested in the field, between species which have contrasting yet still very high foliar Zn concentrations. Within-site variation in foliar Zn concentration and shoot herbivory will also allow the EDH to be tested at a finer scale within a species, for the hyperaccumulator *T. caerulescens* and the accumulator *R. acetosa*.

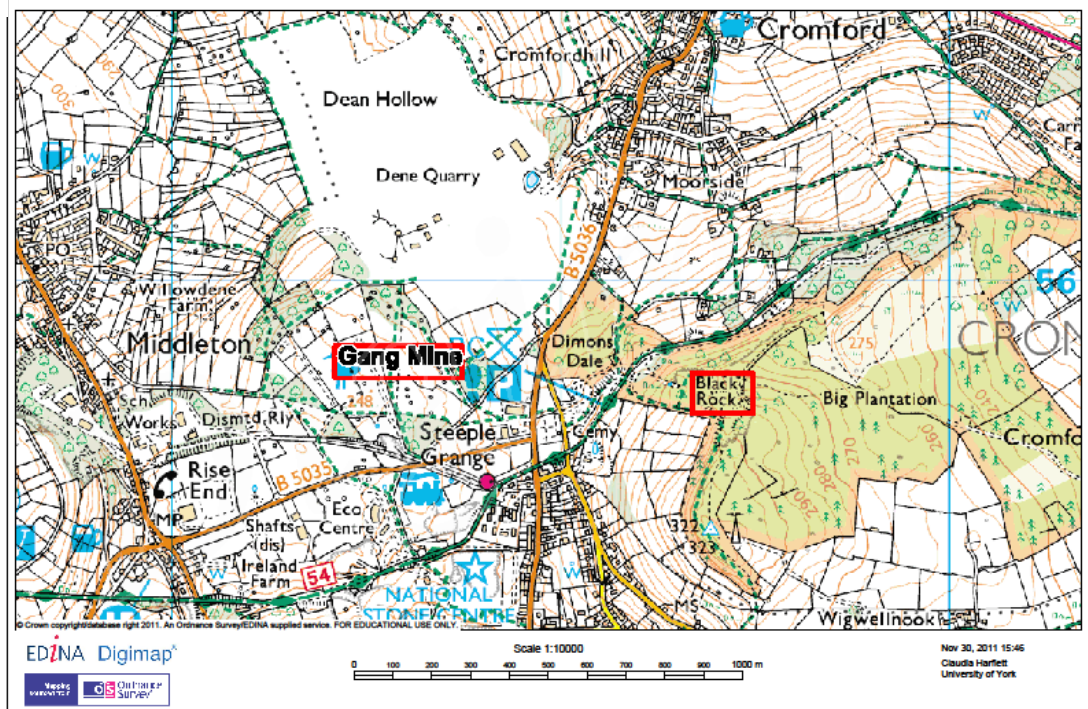
2.1.1 *Study Areas:*

The three metallicolous plant communities in this study were located in the Southern Pennines. Because of their metal-rich rock (mainly Zn, Pb and Cd), areas of the Southern Pennines have been mined for the past 2000 years, leaving behind metal-rich spoils colonised by a unique metal-tolerant plant community (Morrey, Baker & Cooke 1988). Soil Zn and Pb concentrations of 42,000 and 76,500 mg/kg respectively have been recorded (Morrey, Baker & Cooke 1988), approximately 100 times that of non-contaminated soils (Broadley *et al.* 2007).

The three sites surveyed are Black Rock (BR), Clough Wood (CW), and Gang Mine (GM), which all surround abandoned Pb/ Zn mines near Matlock, Derbyshire (**Figure 2- 1**).

Black Rock and Gang Mine are linked by Gang Vein and are approximately 0.5 km apart. Clough Wood is approximately 6 km away from Gang Mine and Black Rock. The study areas are predominately formed of limestone and shales, with Pb- and Zn-rich veins running through the surface bedrock, so that the predominate mining gangue material is calcite producing calcareous grassland conditions (Smith & Bradshaw 1979; Barnatt & Penny 2004). Previous studies on Black Rock and Clough Wood found the hyperaccumulator *T. caerulescens* here growing on soils with extremely high concentrations of Zn, Pb and Cd (**Table 2-1**).

1a



1b

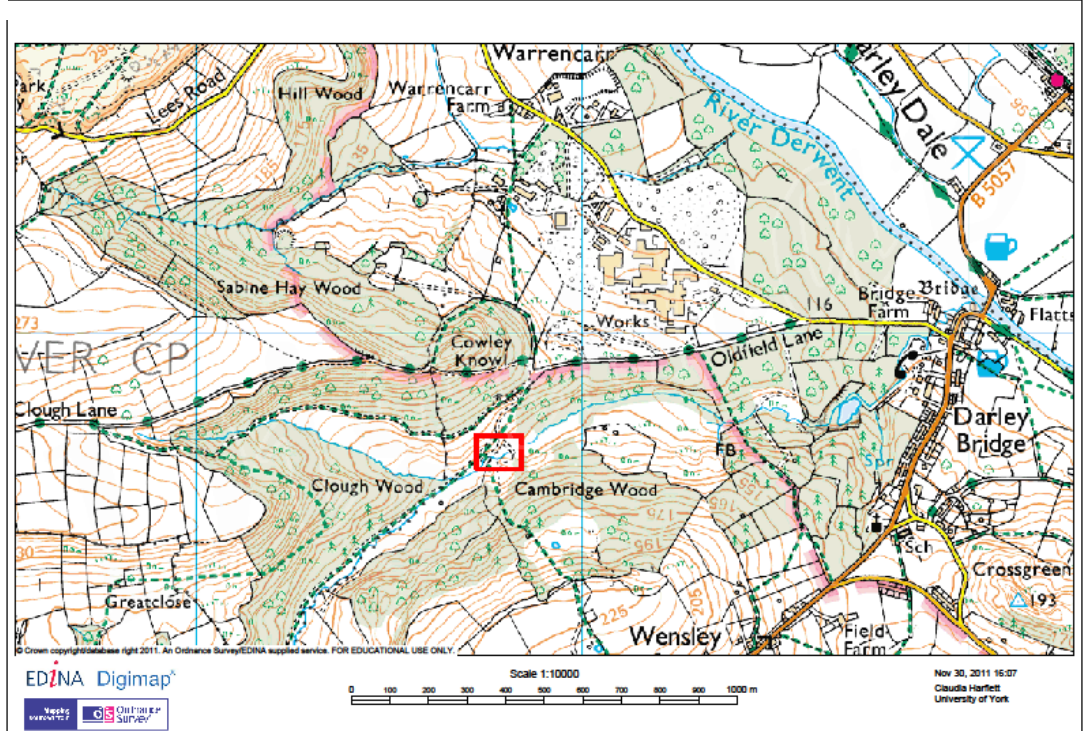


Figure 2- 1 a & b: OS maps of the three sites surveyed in 2010 (highlighted in red). Figure 1a shows Gang Mine and Black Rock and Figure 1b show the site surveyed within Clough Wood.

2.1.1.4 Previous Studies on Field Sites:

Field Site	Citation	Grid Reference	Vegetation above sample	Total Metal concentration (ppm)						pH	% Organic Matter
				Zn	Pb	Cd	Cu	Ca	Ni		
BR	Smith & Bradshaw, 1979	SK 291 557	<i>Festuca rubra</i>	30,000	41,000	NA	153	56,600	NA	6.6	-
BR	Morrey <i>et al.</i> 1988	SK 294 555	NA	11,740	19,427	NA	131	NA	51	<5.0	24.2
BR	Baker <i>et al.</i> 1994	SK 293 557	NA	19,326	90,306	105	NA	NA	NA	7.1	-
BR	Baker <i>et al.</i> 1994	SK 293 557	<i>T. caerulescens</i>	38,000	39,200	348	357	NA	74	7.1	-
CW	Baker <i>et al.</i> 1994	SK 258 618	<i>T. caerulescens</i>	22,900	20,400	270	281	NA	148	7.3	-
CW	Roosens <i>et al.</i> 2003	-	<i>T. caerulescens</i>	25,900	1,777	363	172	NA	123	6.4	-
Normal Soils	Allen, S. E. 1989	NA	NA	20-300	2-20	0.03-0.3	5-100	-	5-500	-	-

Table 2- 1: Summary of Black Rock (BR) and Clough Wood (CW) (Derbyshire, England) soil parameters from previous studies in the literature, and with reference to normal (non-metalliferous soils) in the UK (Allen 1989). No published literature was found on Gang Mine.

Black Rock:

Although not the site of mining works, Black Rock is at the terminus of the heavily worked Godbehere Vein, Gang Vein and Cromford Moor Mines and has nearby shafts and soughs connected to the mineral veins at the nearby Gang Vein ore field (Ford & Rieuwerts 1975). Erosion has caused a landslip to occur below the Millstone Grit escarpment at Black Rock (Ford 2005; Ford 2006), forming a steep slope of scree and mine-tailings, with patches of vegetation amongst and circling the scree. The vegetation becomes more dominant at the base of the slope. The calcareous, neutral pH (sometimes slightly acidic) soil found at both sites supports a variety of metallophytes and calcareous grassland species (Smith & Bradshaw 1979).

Gang Mine:

Gang Mine is a SSSI and has been recorded as an ancient Pb mine since 1652 (Derbyshire County Council 1988b; Ford 2005). The mining spoil is high in Pb and Cd, producing a metallophyte community on the hilly bare spoil heaps and is surrounded by a closed calcareous turf community (Derbyshire County Council 1988b). Although no published studies have recorded the soil Zn and foliar Zn concentrations of this site, a preliminary study in 2009 by the author confirmed the elevated soil and foliar Zn concentrations of *T. caerulescens* and *R. acetosa* here, which were very similar to the concentrations found in the 2010 survey presented in this chapter.

Clough Wood:

The field site at Clough Wood is one of the mining waste tips of Mill Close Mine. This mine was one of the largest, and most certainly the longest running mine in Derbyshire, with records of lead ore being mined from the hillside and surface outcrops until the 17th Century (Ford & Rieuwerts 1975; Brearley 1977). Up until the 20th Century, the Mill Close Mine tips underwent re-dressing (collection of waste rock, and re-processing it) (Edwards, Swinnerton & Hall

1962; Ford & Rieuwerts 1975). This field site is a heterogeneous area, where the remaining mine spoil forms a sharp slope with a boggy area at the bottom. The metallophyte community on the mine spoil is surrounded by nearby woodland, scrub, and a mix of neutral and acidic grassland and is an ecologically important SSSI (Derbyshire County Council 1988a; Barnatt & Penny 2004).

2.1.2 *Chapter Aim:*

By comparing three metalliferous field sites in the South Pennines, the influence of abiotic site differences (total soil Zn concentration, soil pH and organic matter content) on the shoot biomass, Zn concentration factor and foliar Zn concentration of the metallophytes *R. acetosa* and *T. caerulescens* can be determined. By assessing the herbivore damage to the Zn accumulator *R. acetosa* and Zn hyperaccumulator *T. caerulescens* at metalliferous field sites, the potential relationship between foliar Zn concentration and herbivory can be investigated using species contrasting in their foliar Zn concentration and metal tolerance strategies. Within- and between-site analysis will also allow the investigation of fine-scale variation in foliar Zn concentration and herbivory. Therefore this study investigates the EDH in the field between- and within-species, which differ in their foliar Zn concentration and shoot uptake efficiency and assesses how site differences may influence this.

2.1.3 *Null Hypotheses:*

1. There is no statistically significant difference in the soil total Zn concentration, pH and percentage organic matter between the three abandoned Pb/ Zn mine sites.
2. Within a plant species, there is no statistically significant difference in the foliar total Zn concentration, Zn concentration factor, shoot biomass (DW) and

the shoot percentage damage between sites, for either *T. caerulescens* or *R. acetosa*.

3. Within a plant species, there is no statistically significant relationship between soil Zn and foliar Zn concentration, for each site.
4. Within a plant species, there is no statistically significant relationship between foliar Zn concentration and the shoot percentage damage, for any of the sites.

2.2 Methods:

2.2.1 *Sampling Strategy:*

All field sites were surveyed in July 2010. For each site, the bare spoil heaps or scree was examined for the presence of *T. caerulescens*, which was used as a bioindicator of elevated soil Zn concentration. 25 cm² quadrats were placed where there were at least five *T. caerulescens* plants and five *R. acetosa* plants occurring together, on bare or sparsely vegetated scree/ bare spoil. Since both plant species reproduce vegetatively by rammet formation, individual 'plants' were designated by an at least a 10 cm distance between clumps of plants.

Black Rock (Grid Reference SK 293 557) (**Figure 2- 2**) has a long, steep slope, so three transects were run downhill along a continuous axis encompassing the vegetated areas of *T. caerulescens* and *R. acetosa*. A total of 15 quadrats was surveyed. The vegetation seen within each transect consisted mainly of *Festuca rubra*, *Minuartia verna*, mosses and the target species, with the addition of *Euphrasia nemorosa*, *Thymus drucei*, *Fagus sylvatica* and *Betula pendula* further down the slope. The vegetation was sparse at the top of slope and became more dominant at the bottom.

The relief of Clough Wood (Grid Reference SK 258 618) (**Figure 2- 3**) is less steep and more vegetated, and so three parallel transects were run downhill, encompassing the vegetated areas of *T. caerulescens* and *R. acetosa*. A total of nine quadrats were surveyed. Again the vegetation seen within each transect consisted mainly of *Festuca rubra*, *Minuartia verna*, mosses and the target species, with the presence of *Leontodon hispidus* and *Centaurea nigra* at the bottom of the slope and *Betula pendula* flanking the site on the East.

Gang Mine (Grid Reference SK 285 565) (**Figure 2- 4**) has a patchy distribution of bare spoils heaps, sparsely colonised by the target species and *Minuartia verna*, interspersed with mosses and lichens. These spoil heaps are surrounded by vegetation dominated by *Festuca rubra*, *Ranunculus acris*, *Minuartia verna*, *Euphrasia nemorosa*, *Lotus corniculatus*, *Trifolium repens*, *Trifolium pratense* and *Plantago lanceolata*. As there was no natural gradient, transect sampling was not used. Instead 10 bare spoil heaps containing sufficient patches of *T. caerulea* and *R. acetosa* in a 25 cm² quadrat were sampled. The spoil heaps sampled were at least 1 m² and greater than 1 m apart.



Figure 2- 2: Site photo of Black Rock and the area surveyed in 2010 (Grid Reference SK 293 557). Three transects of approximately 1.70, 5.0 and 2.3 m respectively, were run downhill along a continuous axis (total length 47 m), encompassing the vegetated areas of *T. caerulea* and *R. acetosa*. Five quadrats per transect were surveyed.



Figure 2- 3: Site photo of Clough Wood and the area surveyed in 2010 (Grid Reference SK 258 618). Three parallel transects of approximately 23.3, 21.3 and 31.9 m respectively were run downhill, encompassing the vegetated areas of *T. caerulea* and *R. acetosa*. A total of nine quadrats were surveyed.



Figure 2- 4: Site photo of Gang Mine with some of the bare spoil heaps surveyed in 2010 (Grid Reference SK 285 565). 10 bare spoil heaps containing sufficient patches of *T. caerulea* and *R. acetosa*, of at least 1 m² and greater than 1 m apart, were sampled.

2.2.2 *Herbivory Estimations:*

The five *T. caerulea* and five *R. acetosa* plants within each quadrat had their shoot percentage damage from chewing invertebrate herbivores estimated by eye by a single observer. The symmetry and similar size of the rosettes and leaves made this method consistent between plants, species and sites. Leaf damage to the plant was present in many forms - discolouration of the leaves, leaf mining, speckling (black dots on the leaf), chewing, and 'hole-forming'. The last two types were recorded as they are caused by invertebrate herbivore damage (Strauss & Zangerl 2002), whereas the other types were more likely to be caused by pathogens or abiotic stressors. The 'holes' damage consisted of isolated holes within the leaf blade that were completely chewed through, although some had a single translucent layer of epidermis remaining, and was the most common form of damage in *T. caerulea*. Chewing and hole-forming damage were not separated as sometimes they occurred together on the same part of the leaf blade. These categories of damage are most likely to be due to small grazing herbivores such as the Mollusca and small chewing herbivores such as Orthoptera, Coleoptera and Lepidoptera larvae, (Strauss & Zangerl 2002).

2.2.3 *Plant Sample Collection and Preparation:*

The rosettes of the five *T. caerulea* and five *R. acetosa* plants within each quadrat were carefully collected and placed in a polythene bag, separated by site, species and quadrat number. Samples were placed in a cool-box to help prevent desiccation during collection. In the laboratory, plants were carefully washed in reverse osmosis (RO) water, and the flower heads and roots were removed. They were then dried at 60 °C for 48 hours, and left to cool before their dry weight (DW) was recorded. Samples were then ground to a dry, fine powder (<180 µm) using a Fritsch Mini-Pulverisette 23 with stainless steel

accessories (Zn-free with negligible contamination from Ni, Cr and Fe), and stored in polythene self-seal bags until chemical analysis.

2.2.4 *Soil Sample Collection and Preparation:*

For each quadrat, a composite top-soil (0 – 15 cm depth) sample of the area surrounding the plant specimens was collected. For some quadrats the top-soil mainly consisted of thick moss, and because the plant specimens had very small roots (approximately 5 - 8 cm long), which were growing within this moss, it was decided to collect the moss and surrounding top-soil as the 'soil' sample. In the laboratory, the soil samples also had their fresh weights recorded, before drying at 60 °C for 48 hours. Then the dry weight (DW) (g) was recorded. The samples were then sieved through a 2 mm nylon mesh, where a homogenous portion of the < 2 mm fraction (approximately 20 - 30 g) was set-aside for use in pH and loss-on-ignition (LOI) analysis. The remainder of the < 2 mm fraction was ground to a dry, fine powder (<180 µm fraction) using a Tema Mill with agate (SiO₂) accessories. Both fractions were stored in polythene self-grip bags until chemical analysis.

2.2.5 *pH and Percentage Organic Matter Determination:*

The pH of the soil was measured using a pH probe in a 4g to 10 ml soil paste of the < 2mm dried soil fraction, in RO water. The percentage organic matter content was gravimetrically determined by the loss-on-ignition (LOI) technique with the < 2mm pre- dried soil fraction, at 430 °C for 8 hours. LOI provides a reliable and simple estimation of the soil organic matter content, providing that the material is thoroughly dried before use and kept dry throughout, and that the temperature and time of heating used (430 °C for 8 hours) produces minimum loss of CO₂ from soil carbonates such as CaCO₃ (Davies 1974).

2.2.6 *Total Zn Concentration Determination:*

Soil and plant samples contain a large quantity of organic matter, which must be removed before analysis of the inorganic constituents can take place. The total Zn concentration of both soil and plant material was undertaken using 'wet ashing' involving a mixture of nitric, perchloric and hydrochloric acids. This method does not incur the potential loss of volatile elements such as Pb and Cd among others, if further inorganic analysis of the samples is required (Walsh, Gill & Thirlwall, 1997). Where outliers in the soil or foliar Zn concentrations occurred, measurements were repeated using fresh samples to confirm that the concentrations were due to soil heterogeneity or plant within-population variation, rather than sample or reagent contamination.

2.2.6.1 Soil Zn Concentration Analysis:

0.1 g (+/- 0.0010 g) of each soil sample (<180 µm fraction), was weighed out in a randomised order and placed into an 18 mm by 180 mm borosilicate test tube. 4 ml (in excess) of concentrated nitric acid (trace analysis grade, 70 % w/w, Fisher Scientific, Loughborough), followed by 1 ml (in excess) of concentrated perchloric acid (analytic reagent grade, 60% w/w, Fisher Scientific, Loughborough) was added to each test tube. The test tubes were then placed in a deep-holed aluminium hot block, and underwent the following temperature program: three hours at 50 °C, then three hours at 150 °C, then nine hours at 190 °C, followed by three hours at 195 °C. When the program had finished, a dry residue of undigested organic and inorganic material remained. This residue was then leached in 2 ml (gravimetrically calibrated) 5M hydrochloric acid (analytic reagent grade, 36% w/w, Fisher Scientific, Loughborough; diluted in RO water) at 60 °C for 1 hour. 8 ml (gravimetrically calibrated) of RO water was then added, and the mixture vortexed thoroughly before being decanted into a polystyrene 12 ml centrifuge tube, and centrifuged at 2000 rpm for 5 minutes to

separate the sediment from the ionic solution. If the Zn concentration in the solution was above that of the calibration range, then the sample was diluted further in 1 M HCl until it was within the range of the calibration solutions.

2.2.6.2 Foliar Zn Concentration Analysis:

0.1 g (+/- 0.0010 g) of each plant sample was weighed out in a randomised order and placed into an 18 mm by 180 mm borosilicate test tube. 4 ml (in excess) of concentrated nitric acid (trace analysis grade, 70 % w/w, Fisher Scientific, Loughborough) was added to each test tube. The test tubes were then placed in a deep-holed aluminium hot block, and left at 50 °C overnight (approximately 17 hours), to oxidise the majority of the organic matter. The following day 1 ml (in excess) of concentrated perchloric acid (analytic reagent grade, 60% w/w, Fisher Scientific, Loughborough) was added to each test tube. The test tubes were then replaced in the deep-holed aluminium hot block, and underwent the following temperature program: ten minutes at 50 °C, then three hours at 150 °C, then eighteen hours at 190 °C, followed by ten minutes at 195 °C. When the program had finished, a dry residue of undigested organic and inorganic material remained. This residue was then leached in 2 ml (gravimetrically calibrated) 5M hydrochloric acid (analytic reagent grade, 36% w/w, Fisher Scientific, Loughborough; diluted in RO water) at 60 °C for 1 hour. 8 ml (gravimetrically calibrated) of RO water was then added, and the mixture vortexed thoroughly before being decanted into a polystyrene 12 ml centrifuge tube, and centrifuged at 2000 rpm for 5 minutes to separate the sediment from the ionic solution. If the Zn concentration in the solution was above that of the calibration range, then the sample was diluted further in 1 M HCl until it was within the range of the calibration solutions.

2.2.6.3 Blank Correction, Bias and Precision:

For both the plant and soil digestions, reagent blanks (test tubes containing no sample, but undergoing the entire acid digest and dilution procedure) were included approximately every ten test tubes to determine the level of reagent contamination. Matched reagent blank corrections were applied, when required, to the appropriate dilution factor. To determine the relative bias of the chemical analysis (calculated below), oven-dried and homogenously mixed, certified reference materials were included in duplicate and accounted for at least 10 % of the number of samples analysed. To determine the relative precision (calculated as below), randomly selected samples were analysed in duplicate (duplicates coded A and D), again accounting for at least 10 % of the number of samples analysed. Uncertainty of the analytical measurements was deemed to be acceptable if they were both generally within 10% (Gill & Ramsey 1997):

$$\text{Relative Precision (\%)} = 2 \times \text{Median of} \left(\left(\frac{|Duplicate A - Duplicate D|}{\bar{X}} \right) \times 100 \right)$$

$$\text{Mean Relative Bias (\%)} = \text{Mean of} \left(\left(\frac{\text{Concentration}_{\text{measured}} - \text{Concentration}_{\text{certified}}}{\text{Concentration}_{\text{certified}}} \right) \right) \times 100$$

2.2.6.4 Flame Atomic Absorption Spectrometry:

Separate runs were made for the plant and soil samples, due to their different and metal concentration ranges. To determine the total Zn concentration, samples were run on a Flame- Atomic Absorption Spectrometer (F-AAS) (Perkin Elmer Analyst 100), using a Zn hollow cathode lamp (current 15 mA, wavelength 213.9 nm, spectral bandwidth 0.70 nm) and background correction was provided by a deuterium arc lamp. Acetylene (15 psi) and air mix (4: 10) were used for fuel. The AAS was calibrated before use using standards of 0, 1, 3

and 5 µg/ml Zn in 1M HCl (analytical grade, Fisher Scientific, Loughborough), made from a primary stock solution of 1000 ppm Zn in 1M HNO₃ (trace analysis grade, Fisher Scientific, Loughborough). The calibration graph was formulated to have a zero intercept as reagent contamination was corrected for using a calibration blank and the sample matched reagent blanks. A non-linear calibration curve was applied as the dynamic range of the curve was non-linear. Samples that had a Zn absorbance outside or close to the limit of linearity were diluted further to be within the linear range of the calibration curve and re-analysed (Skoog, Holler & Nieman, 1998). Each sample had three instrumental replicates taken at a rate of 1ml/sec. The detection limit and sensitivity of the machine were checked on each run to ensure they were at satisfactory levels.

2.2.7 *Concentration Factor:*

The Concentration Factor (CF) is the quotient of the shoot metal concentration to the soil metal concentration, on a dry weight (DW) basis. A value of 1 shows that the soil and shoot concentrations are similar to each other. Therefore values < 1 show that the shoot concentration is relatively low compared to the soil concentration, and values > 1 show that the shoot concentration is relatively high compared to the soil concentration (Baker 1981). It is calculated as follows:

$$CF (Zn) = \frac{\text{Shoot Zn concentration (mg/kg) (DW)}}{\text{Soil Zn concentration (mg/kg) (DW)}}$$

2.2.8 *Statistical Analysis:*

All data presented in this thesis were statistically analysed using MINITAB (Version 13.1). Assumptions of equal variances and normal distributions for parametric statistics were tested for using Levene's Test and the Kolmogorov-Smirnov test (Zar 2010). Data were transformed if they did not meet these

assumptions, and non-parametric statistics were used in cases where data still did not meet these assumptions after transformation. So that the figures could provide a clear visual representation of the trends, the mean and SE mean were presented for the shoot biomass data instead of the median and IQR.

A one-way ANOVA was used to determine if the mean soil Zn concentration differed between sites. Kruskal-Wallis tests were used to determine if the a) median soil pH and b) percentage organic matter concentration differed between sites. Post-hoc Dunn tests were used to determine which levels were significantly different from each other when $p < 0.05$. General Linear Models (GLMs) were used to determine the effects of Site and Species (fixed factors) and their interaction on the SQRT transformed data of a) foliar Zn and b) concentration factors (CFs). Scheirer-Ray Hare (SRH) tests (non-parametric version of a 2-way ANOVA) were used to determine the effects of Site and Species (fixed factors) and their interaction on a) shoot biomass and b) shoot percentage damage. Post-hoc Tukey tests were used to determine which levels were significantly different from each other when $p < 0.05$. Simple linear regression was used to determine the relationship between the predictor 'soil Zn concentration' and the response 'foliar Zn concentration' for each population of a) *T. caerulea* and b) *R. acetosa*. Pearson's correlation was used to determine the strength of the relationship between the quadrat means of *T. caerulea* foliar Zn concentration and shoot percentage damage. Spearman's rank correlation was used to determine the strength of the relationship between the quadrat means of *R. acetosa* foliar Zn concentration and shoot percentage damage.

2.3 Results:

2.3.1 *Soil Parameters by Site:*

2.3.1.1 Precision and Bias:

For the total soil Zn concentration, the overall precision and overall bias (at 95 % confidence) of the test portions of soil samples and reference materials was well within satisfactory levels at 1.5 % (n = 12) and 5.1 % (n= 7) respectively.

2.3.1.2 Total Soil Zn Concentration:

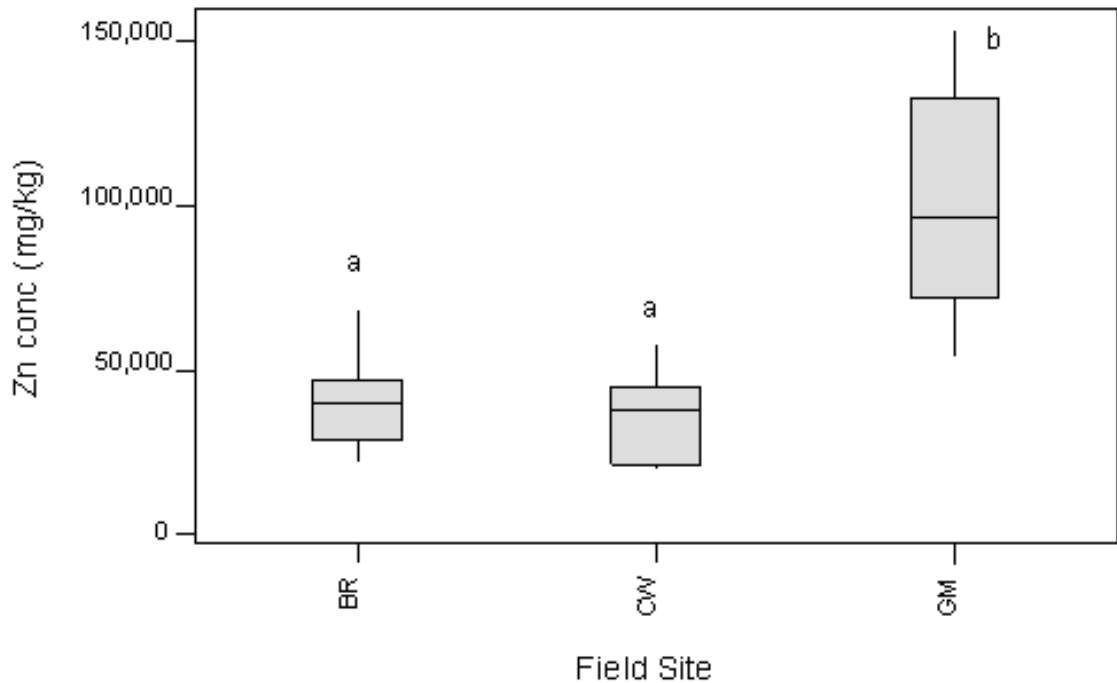


Figure 2- 5: Median and IQR (inter-quartile range) of the soil total Zn concentration (mg/kg) (DW) of Black Rock (n=15), Clough Wood (n=11) and Gang Mine (n=10), Derbyshire, all surveyed in 2010. One way ANOVA on log 10 transformed data: $F = 30.83$, $df = 2, 35$, $p < 0.001$. A post-hoc Tukey test indicates where significant differences lie.

Gang Mine (GM) has a statistically higher soil Zn concentration (median = 96,523 mg/kg,) than Black Rock (BR) (median = 40,248 mg/kg) or Clough Wood (CW) (median = 37,711 mg/kg). However CW and BR were not significantly different from each other (**Figure 2- 5**).

2.3.1.3 Soil pH:

There was no significant difference in the soil pH between the three sites (Kruskall Wallis test adjusted for ties: $H = 2.48$, $df = 2$, $p = 0.289$). The soil pH of the bare mine spoil or scree for all sites ranged between 5.0 and 6.2.

2.3.1.4 Organic Matter Content:

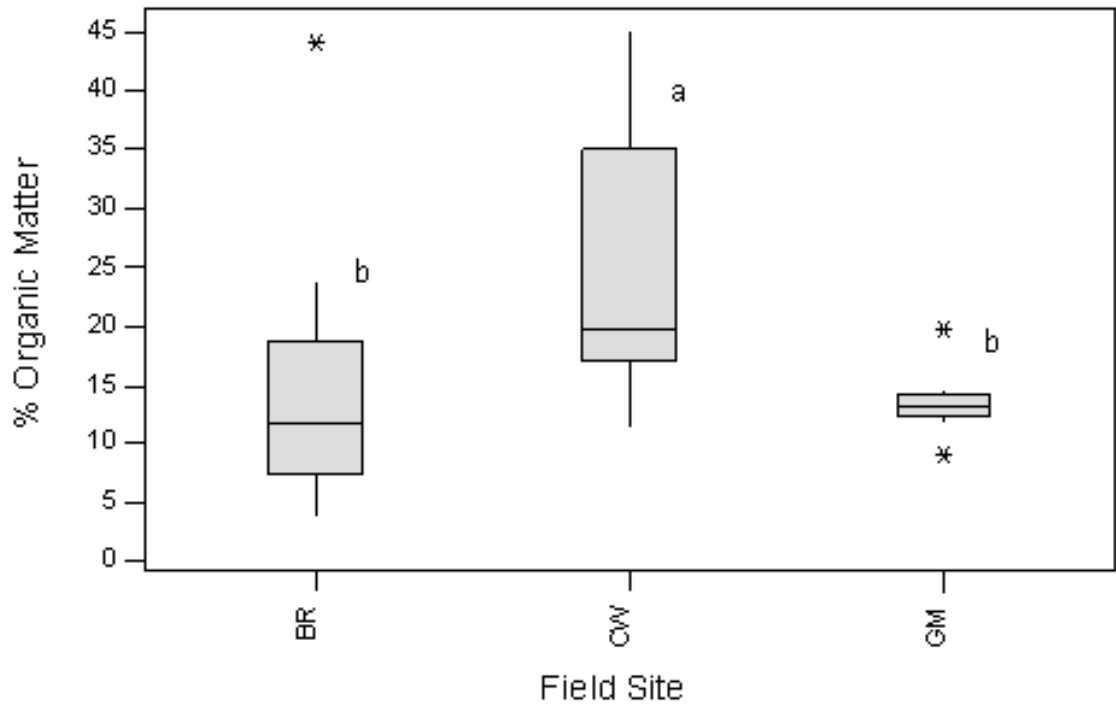


Figure 2- 6: Median and IQR of the % organic matter determined by Lost-on-Ignition, from Black Rock (n = 15), Clough Wood (n = 11) and Gang Mine (n = 10), all surveyed in 2010. Kruskal Wallis (adjusted for ties): $H = 9.50$, $df = 2$, $p = 0.009$. A post-hoc Dunn test indicates where significant differences lie. '*' indicate outliers.

However there was a significant difference in the soil percentage organic matter between the three sites (**Figure 2- 6**). CW soils had significantly higher percentage organic matter content than either BR or GM.

2.3.2 Total Foliar Zn Concentration:

2.3.2.1 Precision and Bias:

The overall precision and overall bias of the test portions of plant samples and reference materials was well within satisfactory levels at 6.5 % (n = 8) and 3.6 % (n = 4) respectively.

2.3.2.2 Total Foliar Zn Concentration

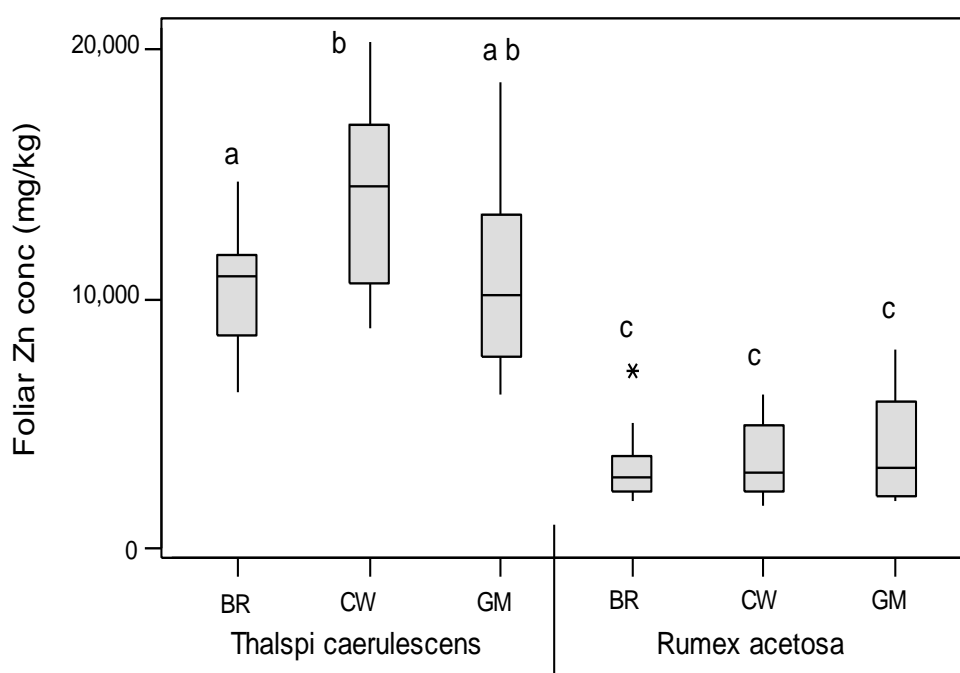


Figure 2- 7: Median and IQR of the foliar Zn concentration (mg/kg) (DW) of *Thlaspi caerulescens* and *Rumex acetosa*. Plants from 3 lead/ zinc mine sites: Black Rock (n=15), Clough Wood (n=11) and Gang Mine (n=10). The statistical output of a GLM on the SQRT transformed data is displayed in Table 2- 2. A post-hoc Tukey test indicates where significant differences lie. '*' indicate outliers.

GLM for SQRT transformed Foliar Zn concentration.						
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Site	2	1258.0	1258.0	629.0	3.10	0.052
Species	1	42654.5	41589.6	41589.6	205.19	<0.001
Site* Species	2	1038.8	1038.8	519.4	2.56	0.085
Error	66	13377.6	13377.6	202.7		
Total	71	58328.9				

Table 2- 2: Statistical output of a GLM, on SQRT transformed foliar Zn concentration data, for two plant species surveyed at three lead/ zinc mine sites.

The foliar Zn concentration was highly significantly different between *T. caerulescens* and *R. acetosa*, and also significantly differed between sites.

However no significant interaction was seen (Figure 2- 7 and Table 2- 2). Site differences were seen for *T. caerulescens*, where plants from the CW site had a significantly greater foliar Zn concentration than BR, but not GM. No site differences were seen for *R. acetosa*.

2.3.3 The Relationship between Foliar Zn and Soil Zn Concentration:

2.3.3.3 *Thlaspi caerulescens*:

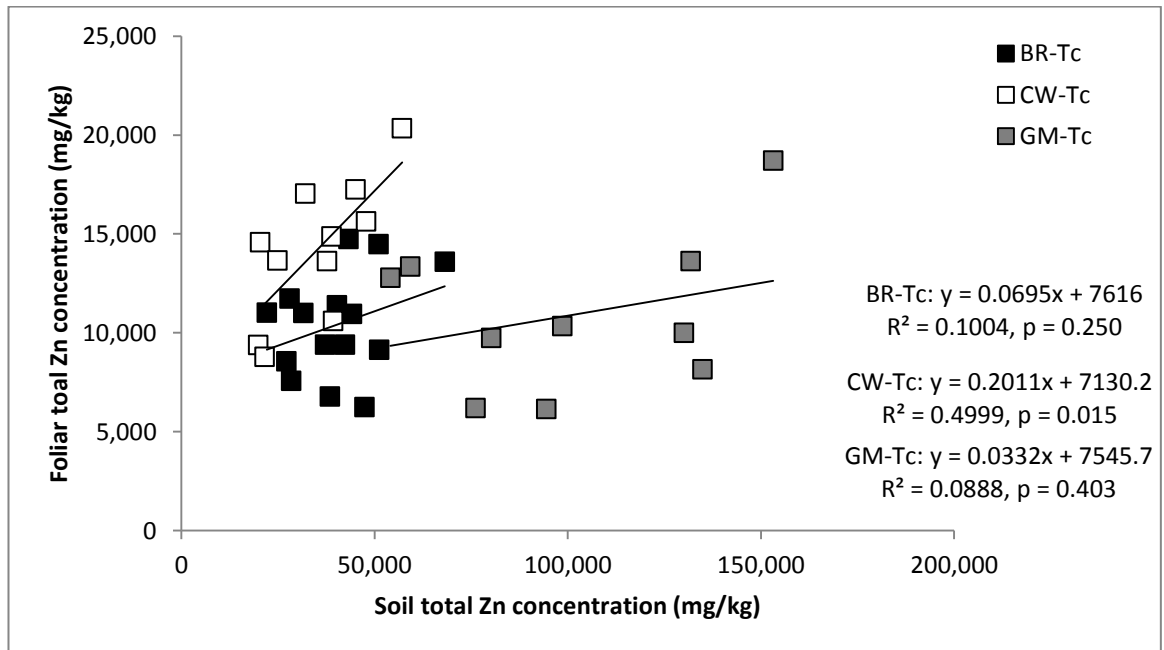


Figure 2- 8: Potential relationship between the total soil and foliar Zn concentration (mg/kg) (DW) of *Thlaspi caerulescens* at Black Rock (BR), Clough Wood (CW), and Gang Mine (GM). The statistical output is presented in Table 2- 3.

Regression analysis results for the relationship between the soil Zn and foliar Zn concentration of <i>Thlaspi caerulescens</i> .						
Site		Coefficient	SE Coefficient	T	P value	R ² (%)
BR	Slope	0.0695	0.0577	1.20	0.250	10.0
	Intercept	7616	2403	3.17	0.007	
CW	Slope	0.2011	0.0670	3.00	0.015	50.0
	Intercept	7130	2474	2.88	0.018	
GM	Slope	0.0332	0.0376	0.88	0.403	8.9
	Intercept	7546	3997	1.89	0.096	

Table 2- 3: Regression analysis of total soil Zn and foliar Zn concentration (DW) relationship for *Thlaspi caerulescens* at three lead/ zinc mine sites.

There is an apparent positive relationship between soil Zn and *T. caerulescens* foliar Zn concentration (Figure 2- 8 & Table 2- 3), but only the plants from CW display a slope coefficient significantly different from zero, whereas the slope coefficients for BR and GM are non-significant and very similar to each other.

2.3.3.4 *Rumex acetosa*:

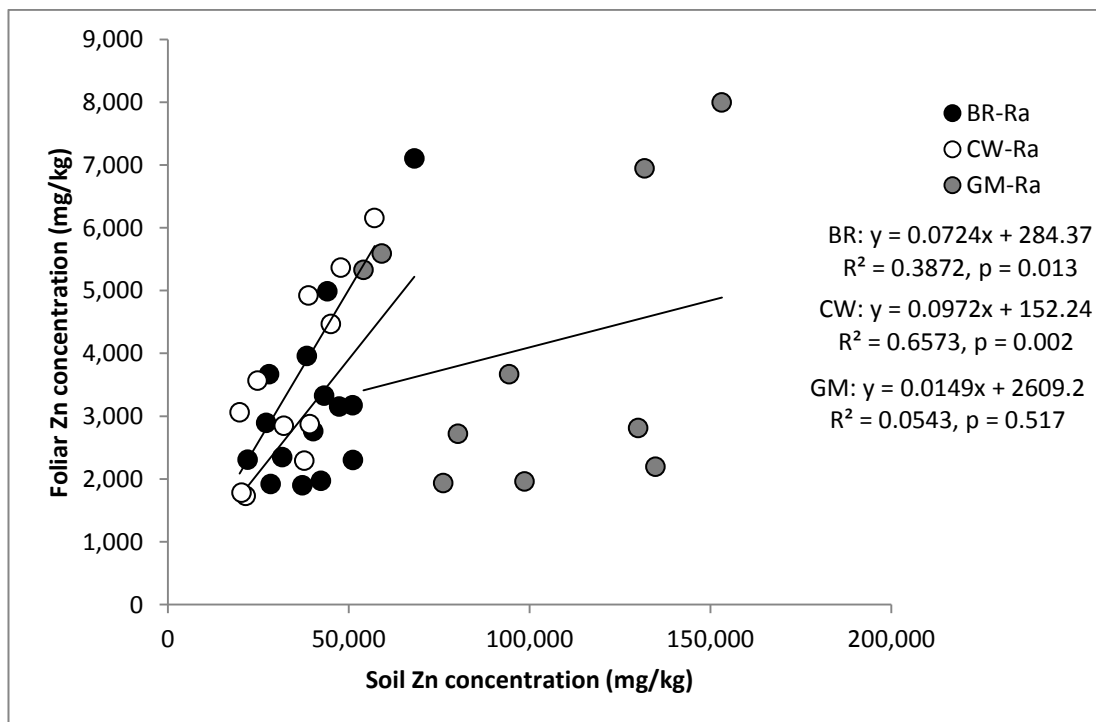


Figure 2- 9: Potential relationship between the total soil and foliar Zn concentration (mg/kg) (DW) in *Rumex acetosa* at Black Rock (BR), Clough Wood (CW), and Gang Mine (GM). The statistical output is presented in Table 2- 4.

Regression analysis results for the relationship between the soil Zn and foliar Zn concentration of <i>Rumex acetosa</i> .						
Site		Coefficient	SE Coefficient	T	P value	R ² (%)
BR	Slope	0.0724	0.0253	2.87	0.013	38.7
	Intercept	284	1053	0.27	0.791	
CW	Slope	0.0972	0.0234	4.16	0.002	65.7
	Intercept	152.2	863.6	0.18	0.864	
GM	Slope	0.0149	0.0220	0.68	0.517	5.4
	Intercept	2609	2336	1.12	0.297	

Table 2- 4: Regression analysis of total soil Zn and foliar Zn concentration (DW) relationship for *Rumex acetosa* at three lead/ zinc mine sites.

There is an apparent positive relationship between soil Zn and *R. acetosa* foliar Zn concentration (**Figure 2- 9 & Table 2- 4**), with plants from BR and CW displaying similar slope coefficients which are significantly different from zero, whereas the plants from GM show a very low and non-significant slope coefficient.

2.3.4 Concentration Factor:

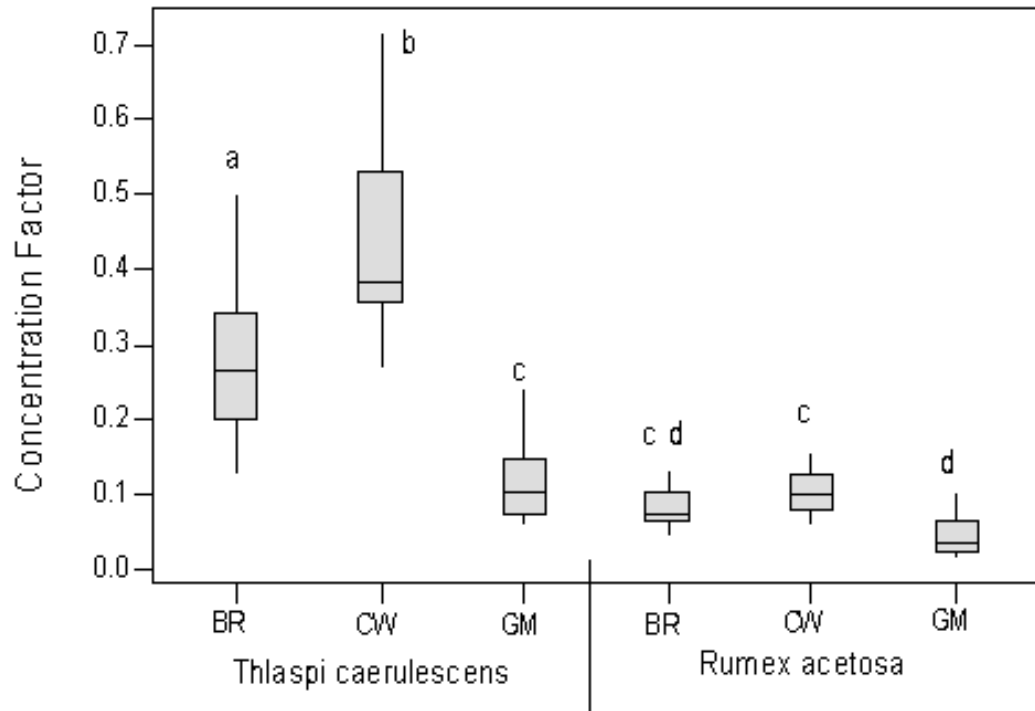


Figure 2- 10: Median and IQR of the concentration factor of *Thlaspi caerulescens* and *Rumex acetosa* populations. Plants from 3 lead/ zinc mine sites: Black Rock (n=15), Clough Wood (n=11) and Gang Mine (n=10). The statistical output of a GLM on the SQRT transformed data is displayed in Table 2- 5. A post-hoc Tukey test indicates where significant differences lie.

	GLM for SQRT transformed Zn Concentration Factor values.					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Site	2	0.48856	0.48856	0.24428	45.61	<0.001
Species	1	1.01967	0.96359	0.96359	179.92	<0.001
Site* Species	2	0.10502	0.10502	0.05251	9.80	<0.001
Error	66	0.35347	0.35347	0.00536		
Total	71	1.96672				

Table 2- 5: Statistical output of a GLM, on SQRT transformed Zn concentration factor values, for two plant species surveyed at three lead/ zinc mine sites.

The concentration factor (CF) was highly significantly different between sites and species, and there was a highly significant interaction between site and species (**Figure 2- 10 and Table 2- 5**). Overall *T. caerulescens* had a higher CF than *R. acetosa*. Plants at CW showed higher CFs than BR, with the plants at GM

having the lowest CF. However this site trend differed between species – for *T. caerulescens* there was a significant difference in the CF between each site, whereas for *R. acetosa* only the CF of the CW plants was significantly greater than the plants of GM.

2.3.5 Shoot Biomass:

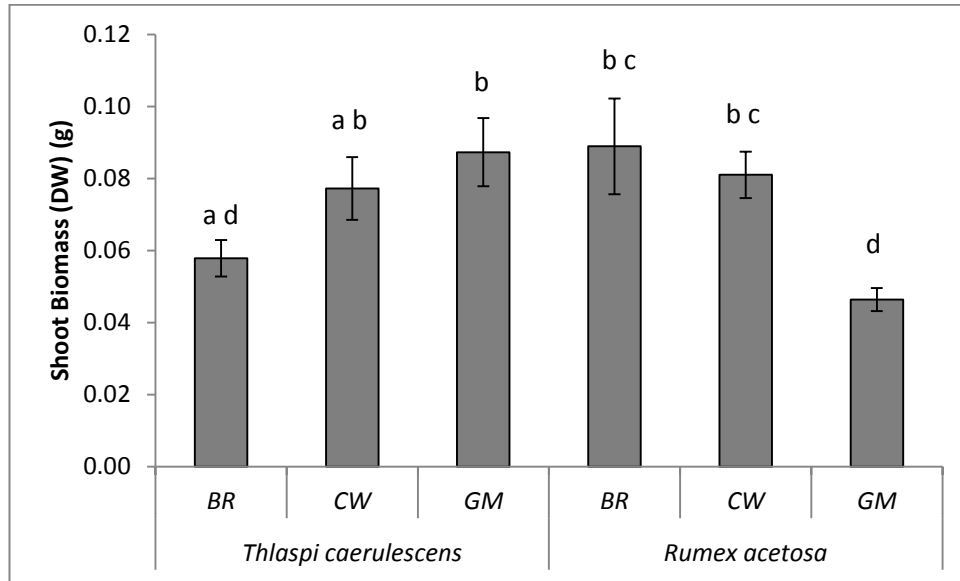


Figure 2- 11: Mean and SE mean shoot biomass (DW) (g) of *Thlaspi caerulescens* and *Rumex acetosa*. Plants from 3 abandoned lead/ zinc mines: Black Rock (n=15), Clough Wood (n=11) and Gang Mine (n=10). The statistical output of a SRH on the ranked data is displayed in Table 2- 6. A post-hoc Tukey test indicates where significant differences lie.

	SRH output of Shoot Biomass (DW).					
Source	DF	Seq SS	Adj SS	Adj MS	Adj SS/ MS total	P value
Site	2	42432	42432	21216	3.918	0.141
Species	1	2717	833	833	0.077	0.781
Site* Species	2	289882	289882	144941	26.767	<0.001
Error	354	3552914	3552914	10036	328.064	
Total	359	3887945		10829.93		

Table 2- 6: Statistical output of a SRH test on the median shoot biomass (DW), for two plant species surveyed at three lead/ zinc mine sites.

There was no significant difference in the median shoot biomass between *T. caerulescens* or *R. acetosa* collected from the bare spoil heaps at the three mining sites (**Figure 2- 11 and Table 2- 6**). The highly significant interaction between species and site shows that the differences in shoot biomass between sites were not the same for both species. For instance the bare spoil heaps at Gang Mine produced the largest shoot biomass for *T. caerulescens* but the lowest for *R. acetosa*. *R. acetosa* at BR and CW had similar shoot biomasses, but *T. caerulescens* plants did not. Instead *T. caerulescens* plants found at CW are larger (although not significantly) than at BR.

2.3.6 Shoot Herbivory and Interaction with Foliar Zn Concentration:

2.3.6.1 Shoot Percentage Damage:

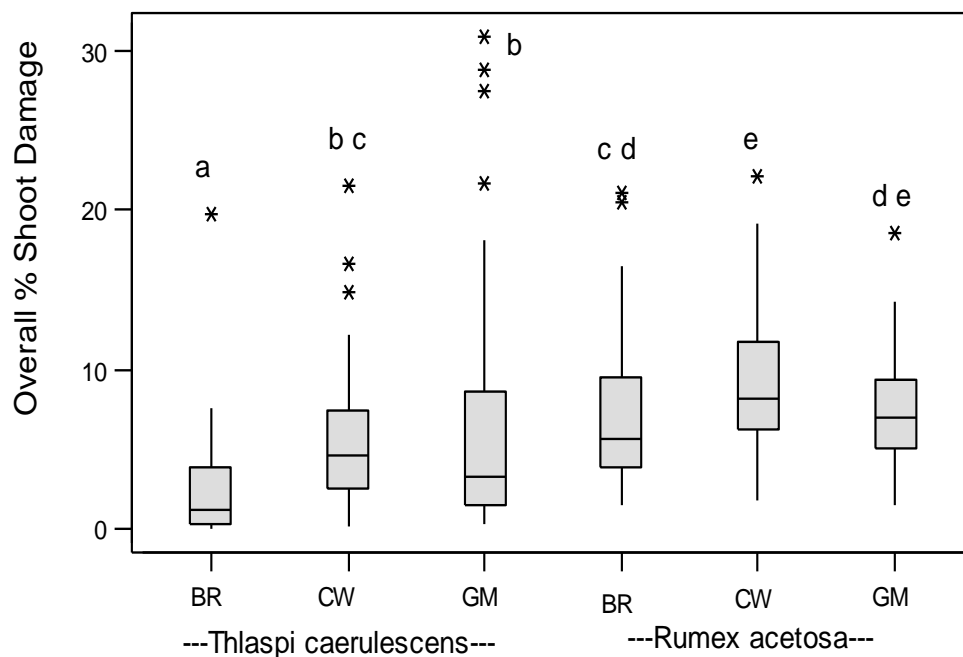


Figure 2- 12: Median and IQR shoot damage (%) of *Thlaspi caerulescens* and *Rumex acetosa*. Plants from 3 lead/ zinc mine sites: Black Rock (n=15), Clough Wood (n=11) and Gang Mine (n=10). The statistical output of a SRH on the ranked data is displayed in Table 2- 7. A post-hoc Tukey test indicates where significant differences lie. “*” indicate outliers.

	SRH output of median shoot percentage damage.					
Source	DF	Seq SS	Adj SS	Adj MS	Adj SS/ MS total	P value
Site	2	42432	42432	21216	3.918	<0.001
Species	1	2717	833	833	0.077	<0.001
Site* Species	2					0.077
		289882	289882	144941	26.767	
Error	354	3552914	3552914	10036	328.064	
Total	359	3887945				

Table 2- 7: Statistical output of a SRH test on the median shoot percentage damage, for two plant species surveyed at three lead/ zinc mine sites.

Overall, shoot damage was very low (**Figure 2- 12 and Table 2- 7**), with the majority of the plants having less than 15 % shoot damage. *R. acetosa* had highly significantly more shoot damage than *T. caerulescens*. However this was also dependent upon site; the plants from CW and GM were significantly more attacked than BR plants overall, particularly for the *T. caerulescens* plants. This site - species interaction did not reach statistical significance however.

2.3.7 Relationship between Foliar Zn Concentration and Herbivory:

2.3.7.1 *Thlaspi caerulescens*:

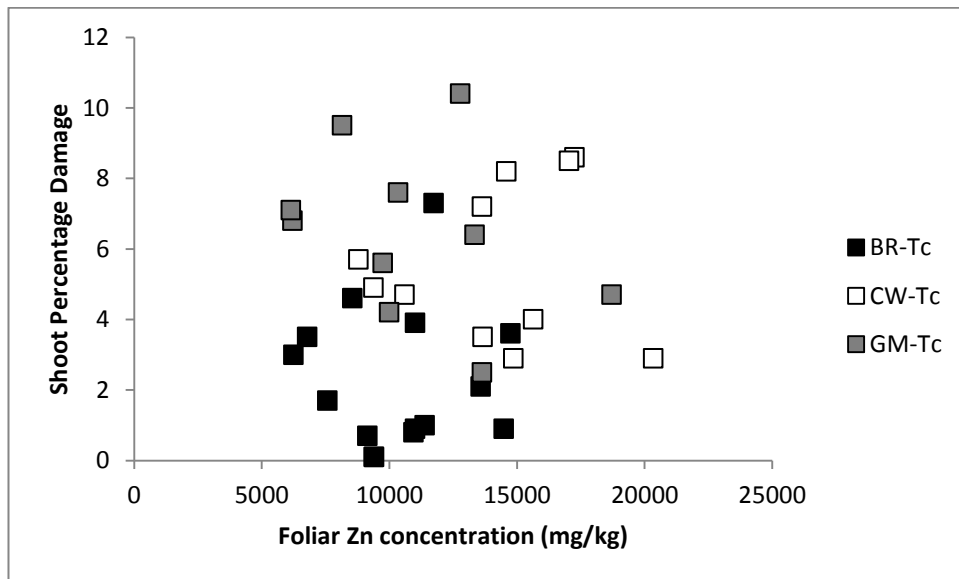


Figure 2- 13: Potential relationship between the quadrat means of foliar Zn concentration (mg/kg) (DW) vs. shoot percentage damage for three *Thlaspi. caerulescens* populations (BR = Black Rock, CW = Clough Wood, GM = Gang Mine). Pearson's Correlation Coefficient: 1) BR-Tc: $r = 0.008$, $P = 0.977$, 2) CW-Tc: $r = 0.051$, $P = 0.822$, 3) GM-Tc: $r = -0.338$, $P = 0.339$, 4) All Sites $r = 0.092$, $P = 0.594$.

The three sites surveyed significantly differed in some aspects of their soil and plant parameters (including Zn concentration) and herbivory levels. The three sites significantly contrasted in their soil total Zn concentration and percentage organic matter hence null hypothesis 1 can be rejected. For *T. caerulea* and *R. acetosa*, null hypotheses 2 and 3 can be rejected as significant site differences were seen in the concentration factors, shoot biomass and percentage herbivore

damage and for the relationship between soil and foliar Zn concentration. Significant site differences were seen in the foliar Zn concentration of *T. caerulescens* but not for *R. acetosa*. However null hypothesis 4 is accepted as no correlation was seen between the foliar Zn concentration and percentage shoot damage for each site, for both *T. caerulescens* and *R. acetosa*.

All sampling points had a soil total Zn concentration of above 25,000 mg/kg and sometimes reached 150,000 mg/kg. Gang Mine produced the significantly highest soil total Zn concentration (median = 96,500 mg/kg), twice that of Black Rock and Clough Wood (medians = 40,200 and 37,700 mg/kg respectively). Gang Mine also has a larger interquartile range (IQR) than Black Rock or Clough Wood, indicating a greater degree of heterogeneity at the site. The organic matter content was additionally very low at all sites (Allen 1989), with Clough Wood having a significantly greater and more patchy distribution of percentage organic matter than Black Rock or Gang Mine, caused by the greater coverage of moss and leaf litter in the Clough Wood soils. However the pH of all sites was statistically similar and indicated slightly acidic to neutral soil conditions at the mining sites.

The foliar Zn concentration of *T. caerulescens* was significantly higher than *R. acetosa* at all sites surveyed, with *T. caerulescens* reaching the hyperaccumulating Zn threshold of 10,000 mg/kg (Baker & Brooks 1989). The high soil Zn concentration produced high foliar Zn concentrations in both metallophyte species, as shown by the positive regression coefficients. Although Gang Mine produced the highest soil Zn concentrations, it did not produce the highest foliar Zn concentrations. This was produced by Clough Wood for *T. caerulescens* (median = 14,584 mg/kg). The foliar Zn concentration for *R. acetosa* did not differ significantly between the sites, and ranged between 1700-6000 mg/kg, sometimes reaching 8000 mg/kg. Additionally Clough Wood showed a

significant regression slope and a significantly higher concentration factor, indicating that of the three sites, both *T. caerulescens* and *R. acetosa* plants growing here are able to take up and tolerate relatively more Zn in their aerial parts. Conversely, the Gang Mine slope coefficient was not significantly different from zero and had a significantly lower concentration factor, indicating that plants here restrict the movement of Zn into their aerial parts to greater extent. The biomass of both species was very small (generally under 0.2 g) and similar between all sites. Additionally *R. acetosa* had a highly significantly greater degree of shoot damage than *T. caerulescens* - the overall herbivore damage to *R. acetosa* was less than 15 % and for *T. caerulescens*, less than 10 %. This range was similar between sites, though the Black Rock site had the significantly lowest percentage damage. No correlation was found between the foliar Zn concentration and percentage damage for each site or for all sites combined.

2.4 **Discussion:**

Chemical analysis of the soil total Zn concentration gave very high concentrations, typical of mining sites in the Pennines (Baker, Reeves & Hajar 1994) and similar to the previous surveys of these sites (see **Table 2-1**). Black Rock and Clough Wood had similar concentrations of soil total Zn, whereas Gang Mine had two to three times this amount. These site differences are likely to be due to the underlying geology and natural and anthropogenic processes occurring at the sites mentioned above (Barry & Clark 1978). The mining sites surveyed produced stressful conditions for the growth of metal-tolerant populations of *T. caerulescens* and *R. acetosa*, as the shoot biomass of the plants was very small, typically less than 0.2g (DW) per plant. Although the shoot biomass was not significantly different between *T. caerulescens* and *R. acetosa*, pot trials have shown contrasting effects of soil Zn concentration on the

biomass of the two species. In separate pot trials manipulating the soil Zn concentration, *T. caerulescens* either did not produce a change in shoot biomass, or increased its shoot biomass with increasing soil Zn concentration (Escarré *et al.* 2000; Dechamps *et al.* 2007). In contrast, Barrutia *et al.* (2009) found that the shoot biomass of metallicolous accessions of *R. acetosa* was significantly reduced as soil metal (Zn, Pb and Cd) concentration increased, from approximately 17 g in the control treatment to 2 g in the pure mine tailing soil, i.e. a six-fold reduction. Additionally, although the addition of fertilizer did stimulate both metallicolous and non-metallicolous plant growth within a soil treatment, the addition of fertilizer did not affect the trend of decreasing shoot biomass with increasing soil metal (Zn, Pb and Cd) concentration. Thus metal (Zn, Pb and Cd) toxicity was thought to be the dominant limiting factor to shoot growth. The direction and magnitude of this trend indicates that the shoot biomass of *R. acetosa* is more affected by soil Zn concentration than *T. caerulescens*, and thus the latter has a greater metal tolerance (Baker 1987). This could be linked to the metallicolous specialisation of these two species – in the UK *R. acetosa* is usually found on neutral grassland rather than metalliferous soils and is classed as a pseudometallophyte, whereas populations of *T. caerulescens* are only found on metalliferous soils and is thus classed as an obligate metallophyte (Baker & Proctor 1990). Therefore metal tolerance at these sites has potentially a greater cost to the pseudometallophyte compared to the obligate metallophyte (Baker 1987). There was also no significant difference in shoot biomass between sites; although a significant interaction was seen. For *R. acetosa* shoot biomass was similar between Black Rock and Clough Wood but significantly smaller for Gang Mine. This may be linked to abiotic differences between the sites, the increased soil Zn concentration and decreased organic matter content of the Gang Mine soil, coupled with *R. acetosa* lower metal tolerance could reduce the shoot biomass here (Barrutia *et al.* 2009). Organic matter in mine spoil is a slow-release source of essential plant nutrients which are very limited on

metalliferous soils, such as nitrogen and phosphorous, and prevents leaching of these elements and retention of moisture (Tordoff, Baker & Willis 2000).

Organic matter can also contain a higher quantity of soil biota which aids vegetative growth through improved soil structure and increasing the soil nutrient availability (Tordoff, Baker & Willis 2000). However for *T. caerulescens*, the shoot biomass was more similar between sites, although smallest at Black Rock, demonstrating that site differences may have a contrasting or less of an effect on the shoot biomass of the obligate metallophyte.

The foliar Zn concentration of *T. caerulescens* at all field sites was extremely high, ranging from 6000 – 20,000 mg/kg. Like other pseudometallophytes (McGrath & Zhao 2003), *R. acetosa* had a far lower foliar Zn concentration compared to *T. caerulescens* in the field, approximately $1/10$ to $1/5$ of *T. caerulescens* levels. The range seen in metalicolous populations of *R. acetosa* (1700 – 8000 mg/kg) was similar to other published studies (Shimwell & Laurie 1972; Johnston & Proctor 1977). The efficiency of soil to shoot uptake was investigated using the concentration factor (CF). Values < 1 show that the shoot concentration is relatively low compared to the soil concentration and that the plant is strongly restricting metal transport from the roots to the shoots, whereas values > 1 indicate active Zn transport mechanisms from the soil and roots to the shoots (Baker 1981). Although *T. caerulescens* is classed as a hyperaccumulator due to its $> 10,000$ mg/kg foliar Zn concentration (Baker & Brooks 1989), *T. caerulescens* median CF was ≤ 0.4 for all sites, indicating that *T. caerulescens* in the field is potentially restricting the Zn uptake and shoot transport. Significantly lower CF values were also seen for *R. acetosa* (median ≤ 0.1 for all sites). This demonstrates that both species were potentially restricting Zn transport into their shoots as a metal tolerance strategy. The most common mechanism of plant adaptation to metal toxicity is an increased restriction of root metal uptake and restricted shoot transport (Briat & Lebrun 1999; Macnair

2003; Zhao, Lombi & McGrath 2003). This is due to saturation of Zn shoot-uptake and thus reduced root-to-shoot transport, or a down-regulation of Zn root-uptake and thus reduced root-to-shoot transportation (Pence *et al.* 2000). Generally as a hyperaccumulator, *T. caerulescens* has an active uptake mechanism(s) for acquiring Zn from low-Zn soil (Whiting *et al.* 2001) and can additionally forage for Zn in Zn-rich patches (Haines 2002b), demonstrating a potential requirement for maximising Zn uptake (Whiting *et al.* 2000). However, there is an indication that at the total soil Zn concentrations seen in this study, *T. caerulescens* may actually restrict Zn shoot uptake, although to a lesser degree than *R. acetosa*.

Site differences were also seen in the concentration factors and the slope coefficients for both plant species. Plants growing in the higher soil Zn concentration of Gang Mine had lower CFs and slope coefficients, suggesting that the movement of Zn from the soil to the shoots was restricted. However this restriction may be due abiotic site differences in the bioavailability of Zn, or due to possible site differences in the plants' tolerance mechanisms.

Although a soil may contain a large quantity of metal ions, these ions may not be in a form that can be readily taken up by plants. The bioavailable fraction is the concentration of the metal in the soil that is free to be taken up by the plant - for Zn approximately < 10% is bioavailable (Broadley *et al.* 2007). Zn is distributed in the soil between different types of 'fractions' or pools, the most mobile pools being water-soluble, exchangeable (adsorbed to clay and organic matter particles) and bonded to organic complexes such as chelates (Alloway 1995; Kiekens 1995). This is affected by many abiotic factors such as the chemical form of the metal, the soil type and structure, and the soil chemistry e.g. the soil pH, redox potential, cation exchange capacity (CEC) and the organic matter content (Lindsay 1972; Ernst 1996; Tordoff, Baker & Willis 2000;

Whiting *et al.* 2001; Wong 2003). Dead leaves containing elevated levels of soluble Zn may also contribute to the bioavailable fraction to the plants (Schwartz *et al.* 2001). Therefore determining the most appropriate chemical method for assessing the bioavailable fraction of Zn is complicated. Additionally, other factors, for instance the nutrient and drought status of the soil and potential microbial interactions, are also known to influence the metal tolerance and foliar metal concentration (Shen, Zhao & McGrath 1997; Tordoff, Baker & Willis 2000; Whiting *et al.* 2001; Assunção, Schat & Aarts 2003; Yang *et al.* 2005; Hildebrandt, Regvar & Bothe 2007).

For most metals, including Zn, a low pH and a high organic matter content increases their bioavailability (Lindsay 1972; Kieken 1995; Broadley *et al.* 2007). In this study the soil pH did not differ between sites, ranging between 5.0 and 6.2, and is typical of neutral grasslands (Alloway 1995). The organic matter content however ranged from 5 to 45 %, and was much higher and more variable at Clough Wood compared to Gang Mine. The influence of organic matter on the metal availability of the soil and metal tolerance of the plant is complex. Higher levels of soil organic matter generally increases the CEC of the soil, (i.e. the total amount of exchangeable cations that a given quantity of soil can absorb), and also adsorb the free metal-ions by forming chelate complexes (Alloway 1995). Although increased organic matter is thought to increase the bioavailability of Zn due to the subsequent decrease in pH and the formation of soluble Zn complexes with fulvic acid, it can also form insoluble metal complexes through binding with humic acids (Lindsay 1972), thus reducing the bioavailability of metals by forming insoluble metal-complexes, and thus increasing plant survival on metal-enriched soil (Tordoff, Baker & Willis 2000; Shuman, Dudka & Das 2001; Pilon-Smits 2005). However in the rhizosphere, other organic compounds such as citrate or malate may mobilise metals which are present in the soil, and form other metal-complexes which can be taken up

by the roots and into the plant (Mathys 1977; Alloway 1995; Pilon-Smits 2005). Additionally, the soil biota in organic matter may include chemolithotrophic bacteria which acidify the soil and enhance metal mobility and/ or mycorrhizal fungi which can enhance metal uptake by binding metals to fungal hyphae, thus preventing the translocation of metals in the roots to the shoots (Ernst 1996; Tordoff, Baker & Willis 2000; Hildebrandt, Regvar & Bothe 2007).

Therefore the influence of abiotic site differences is complex and a variety of abiotic factors may have produced the site differences seen in the CFs of both species and the foliar Zn concentration of *T. caerulescens*. For *R. acetosa*, the foliar Zn concentration did not differ significantly between sites even though the plants at Clough Wood again displayed the highest CF and Gang Mine the lowest CF. Therefore, although site differences similarly affect the CF of both species, they do not seem to influence the foliar Zn concentration of *R. acetosa* as much as they do for *T. caerulescens*. The site differences seen could be abiotic differences mediated by potential population differences. For example plants at Gang Mine may have also developed tolerance mechanisms which restrict Zn uptake to a greater extent than Clough Wood because of the greater total concentration of Zn ions in the soil. Therefore manipulative experiments are required to determine if population differences in metal uptake occur and how this influences the foliar Zn concentration of these two species.

The Elemental Defence Hypothesis (EDH) suggests that the foliar metal concentration is influenced by herbivory as well as soil concentration. It proposes that higher foliar metal concentrations (like those seen in hyperaccumulators compared to non-hyperaccumulators) on metalliferous sites have been selected for as an herbivore defence strategy (Boyd & Martens 1992). Both plant species accumulated large quantities of foliar Zn. Using the foliar Zn concentration values, *R. acetosa* can be classed as an Zn accumulator, and *T.*

caerulescens a Zn hyperaccumulator, as foliar Zn concentrations greater than 3000 mg/kg in Zn-rich soils are considered unusual (Reeves & Baker 2000). This study's findings go some way to support the EDH, as overall *T. caerulescens* was significantly less damaged than *R. acetosa*. The large difference in the shoot percentage damage between species could be due to the five-fold difference in foliar Zn concentration, and potentially provides support for the EDH, at least when comparing *R. acetosa* with *T. caerulescens*. This conclusion is supported by the only field study which compares hyperaccumulators with non-hyperaccumulating species in a metalliferous environment. Galeas *et al.* (2008) found a lower arthropod load on Se-hyperaccumulating plants compared to non-hyperaccumulating plants growing in a Se-rich environment. Those arthropods which were found on the Se hyperaccumulators had a three-fold higher body Se concentration compared to those found on non-hyperaccumulating plants, therefore displaying some degree of specialisation on Se-hyperaccumulating plants.

However the influence of the foliar Zn concentration on herbivore deterrence cannot be fully determined in this field survey as the influence of other aspects of plant palatability, such as organic defences and nutritional quality, were not assessed. Therefore the influence of foliar Zn concentration as a direct herbivore deterrence mechanism and the physiological effects of growth under elevated soil metal concentrations on other aspects of plant palatability, such as nitrogen content or organic defences, are unknown. Although the EDH can be supported (directly or indirectly) when contrasting the absolute foliar Zn concentrations and shoot damage of *T. caerulescens* with *R. acetosa*, the EDH is not supported when considering variation in the foliar Zn concentration between sites. No relationship was found between the foliar Zn concentration and shoot damage levels for any site or species, therefore small differences in the foliar Zn concentration (produced by site or genetic differences) do not seem influence

the level of herbivory. Additionally, the Clough Wood *T. caerulescens* population have the highest foliar Zn concentrations but also are relatively more attacked. The lack of a relationship between foliar Zn concentration within a plant species and levels of herbivory may be due to the low abundance of herbivores of herbivores at these metalliferous sites. Shoot herbivory was very low at the mining sites, usually 5 - 10%, with some plants at BR experiencing less than 1 % chewing damage. Personal observations of the sites found very few herbivores on the surrounding vegetation and no chewing herbivores were observed feeding on the plants collected. This suggests the selective pressure to increase uptake as a deterrent to herbivory is low and that other factors may drive differences in foliar Zn levels between plants and sites. This is supported by Noret *et al.* (2007b) field study which found that metalliferous environments had a lower abundance of herbivorous gastropods. The low herbivore pressure is probably due to both the influence of metal toxicity on gastropods (Laskowski & Hopkin 1996b; Laskowski & Hopkin 1996a; Gomot-de Vaufleury & Kerhoas 2000; Notten *et al.* 2006b) and other unfavourable environmental conditions on metalliferous soils (Noret *et al.* 2007b).

As the foliar Zn concentration *per se* cannot explain the significant differences in damage seen within and between sites, it is suggested that that other site factors relating to the herbivores and plants are probably more relevant to the degree of shoot damage seen. Differences could include the type of chewing herbivore and their behaviour and abundance, other plant defences, differences in plant nutritional quality and the presence of other heavy metals, such as Pb and Cd. The Pb and Cd concentration of both plant species (but not the soil concentration) was determined for the Black Rock and Clough Wood sites in a pilot study (2008): the mean *R. acetosa* Pb concentration for Black Rock and Clough Wood respectively was 932 and 80 mg/kg, and for *T. caerulescens* 1560

and 276 mg/kg. The mean *R. acetosa* Cd concentrations for Black Rock and Clough Wood respectively was 11 and 7 mg/kg, and for *T. caerulescens* 102 and 215 mg/kg. Thus the presence of other heavy metals in the shoots, and the plant's response to growth in Pb and Cd contaminated soils could also influence palatability, especially Pb in this case. As the soil Pb and Cd concentration was not determined at the time, this alongside the influence of Pb and Cd on the palatability of both *R. acetosa* and *T. caerulescens* could be carried out in further work. To determine which of the above mentioned factors are the most important in influencing plant palatability in the field, reciprocal transplant experiments involving the above metalicolous populations, and well as selecting a suitable non-metallicolous population of *R. acetosa* growing nearby on mineral soils could be utilized in further work. Unfortunately non-metallicolous populations of *T. caerulescens* have not been found in the U.K (Ingrouille & Smirnoff (1986) to allow reciprocal transplantation.

Using transplantation experiments, the Noret *et al.* (2007b) field study supported this conclusion, which investigated the role of Zn hyperaccumulation and another type of plant defence, glucosinolates, in field populations of *T. caerulescens* from metalliferous and non-metalliferous environments. Contrary to the EDH, transplantation experiments showed that non-metallicolous populations were significantly eaten less compared to metalicolous populations when grown at non-metalliferous and metalliferous sites. The lower herbivore pressure found at metalliferous sites in the Noret *et al.* (2007b) field study was also coupled with a lower glucosinolate concentration in the metalicolous populations. Therefore, it seems that the conditions present at metalliferous sites directly and indirectly deter herbivory, partially from elevated foliar Zn concentrations and partially from other unfavourable environmental conditions, thus producing generally low levels of damage. Noret *et al.* (2007b) concluded that this could have selected for lower

glucosinolate concentrations in metalicolous populations, and thus a higher palatability when transplanted in the more herbivore-abundant uncontaminated environments. In non-metalliferous environments, plants do not have the potential advantage of a low-cost elemental defence, and therefore may invest more in organic defences. This demonstrated a potential trade-off between the level of elemental and organic defences in metalicolous plants – low herbivore abundance could be deterred by the elevated foliar metal concentration, resulting in a decrease in potentially higher-cost organic defences. Herbivore pressure may additionally influence the degree of and interaction between inorganic and organic defence compounds (the 'Joint Effects Hypothesis' (Boyd 2007)), although the full implication of this is still unknown and remains an important aspect of understanding hyperaccumulation and the Elemental Defence Hypothesis (Rascio & Navari-Izzo 2011). Therefore the potential trade-off between elemental and organic defences and its implications for herbivory levels and the EDH in metalicolous communities warrants further investigation.

2.5 Conclusion:

In the field, between-species differences were seen in the foliar Zn concentration, CF, and shoot percentage damage of the Zn hyperaccumulator *T. caerulescens* and the Zn accumulator *R. acetosa*. *T. caerulescens* had a higher foliar Zn concentration, was more efficient at transporting Zn from the soil to the shoots, and had lower shoot damage than *R. acetosa*. This provided support for the EDH between species. However, the variation in foliar Zn concentration within a site was not related to the shoot damage for either species, hence finer-scale differences in foliar Zn concentration do not seem to have an effect on herbivory. Between-site differences were also seen for both species for the shoot biomass, CF, and shoot damage, and for foliar Zn concentration in *T.*

caerulescens. Although site differences had contrasting effects on the foliar Zn concentration and shoot biomass, and thus metal tolerance of *T. caerulescens* and *R. acetosa*, similar trends in the concentration factor between sites were seen for the two species. These site differences could also be mediated by potential population differences as well. Manipulative experiments are required to investigate further the complex interactions between the soil metal concentration, the metal tolerance of the plant and its ability to defend itself from herbivory.

Chapter 3: Two Pot Trials investigating Shoot Differences and Herbivore Preference in Metallicolous Populations of *Rumex acetosa*, through the Manipulation of the Foliar Zinc Concentration using Population and Growth Medium Differences.

3.1 Introduction:

In the field (**Chapter 2**) the metallophytes *Thlaspi caerulescens* and *Rumex acetosa* both displayed elevated foliar Zn concentrations, to hyperaccumulator and accumulator concentrations respectively (Reeves & Baker 2000). Low shoot damage levels (< 15%) were additionally seen, significantly less in *T. caerulescens* compared to *R. acetosa*, supporting the Elemental Defence Hypothesis (EDH) when comparing between species. However although site variation in herbivory was seen in the field, it did not correlate with foliar Zn concentration, suggesting that the foliar Zn concentration is not directly related to the palatability of the plant, and that the EDH is not supported within a species. Since many abiotic and biotic factors, such as nutritional quality, herbivore abundance and other plant defences, interact with each other to influence the palatability of shoot tissue, manipulative experiments under controlled conditions are required to identify the influence of foliar metal concentration on palatability.

Since the Elemental Defence Hypothesis was proposed by Boyd & Martens in 1992, a variety of manipulative experimental studies on hyperaccumulating plants have been carried out (reviewed in Boyd (2007) and in Vesik & Reichman (2009)). Performance trials have demonstrated reduced survival, biomass and longer time to reach pupation in herbivores fed metal-rich leaves compared to

control leaves (Ernst, Schat & Verkleij 1990; Boyd & Martens 1992; Boyd & Martens 1994; Boyd & Moar 1999; Noret *et al.* 2007a) and in artificial diets manipulating the metal concentration (Behmer *et al.* 2005; Coleman, Boyd & Eubanks 2005). However in order for elevated shoot metal concentration to benefit the plant, the plant must be able to survive herbivore attack and produce offspring; thus deterrence of herbivory through reduced palatability and sub-lethal effects is a better indicator of an selective advantage (Pollard 1992; Boyd 1998; Macnair 2003). Preference or choice trials provide a better indication that deterrence is a selective advantage (i.e. adaptive), but with caution as the advantage seen may not be for the original reason that the trait was selected for (i.e. exaptation) (Boyd 2004). The majority of preference trials investigating the EDH use hyperaccumulating plant species, and manipulate the foliar Zn concentration by the soil metal concentration. Generally, herbivores prefer the low foliar Zn choice using this method, but individual preference trials have revealed much variation in wherever a preference or deterrence for high metal diets was observed, possibly due to the influence of herbivore type and feeding guild, the few number of plant species studied (Boyd 2007; Vesik & Reichman 2009), the metal investigated (Vesik & Reichman 2009), the contrast in the foliar metal concentrations used and the potential interaction with other plant defences (Boyd 2007). Therefore different investigative approaches are required to fully understand the influence of foliar metal concentration on herbivory. Boyd (2007) concluded his review with two points – 1) what is the minimum elemental concentration required for defence and 2) how do organic defence compounds interact with elemental ones to produce overall deterrence? This chapter explores these questions by using the Zn accumulator *R. acetosa* which showed site differences in palatability in the field (**Chapter 2**).

Firstly, research using plants with foliar metal concentrations lower than hyperaccumulator levels is needed to fully understand the ecological processes and evolution of metal tolerance and elemental defence (Boyd 1998; Boyd & Moar 1999; Boyd 2004; Boyd 2007). A relatively small number of laboratory studies have found that plants with foliar Ni, Se and Zn concentrations lower than those in hyperaccumulating plants are able to deter herbivory (Hanson *et al.* 2003; Hanson *et al.* 2004), reduce herbivore performance (Boyd & Moar 1999; Hanson *et al.* 2004; Noret *et al.* 2007a), or produce fatal effects in herbivores (Trumble, Kund & White 1998; Boyd & Moar 1999), supported by experiments using artificial diets (Vickerman & Trumble 1999; Coleman, Boyd & Eubanks 2005). Thus the deterrence of herbivores from elemental defence may be more widespread than previously thought (Boyd 2007).

Secondly, the majority of preference studies have altered the foliar Zn concentration of the plant used in choice trials externally by using high and low soil metal concentrations. However, the metal concentration of the growth medium is likely to influence plant palatability through other physiological effects aside from increasing the foliar metal concentration of the plant (Boyd 2007), such as metal-stress effects and toxicity response mechanisms to iron and phosphorus inhibition, chlorosis and stunted growth (Foy, Chaney & White 1978) and cellular breakdown, reduced starch content and decreased water content (Sridhar *et al.* 2005). In the Ni hyperaccumulator *Streptanthus polygaloides*, soil metal concentration has been found to additionally affect the plant moisture content and macro-nutrient concentrations (Martens & Boyd 1994). In the Zn hyperaccumulator *T. caerulescens*, the levels of the organic defence compounds glucosinolates (Tolrà *et al.* 2001), and the root foraging behaviour and morphology (Schwartz *et al.* 1999) are altered by the soil Zn concentration. Additionally Tolrà *et al.* (2005) found the soil aluminium (Al) concentrations significantly affected the type and concentration of root and

shoot organic compounds in *R. acetosa*, which could be involved in root Al stress and Al-tolerance. Therefore when investigating the effect of foliar metal concentration on herbivory, the effects of foliar Zn concentration needs to be untangled from the other plant physiological aspects produced by manipulating the soil Zn concentration.

A more direct method of investigating the influence that foliar metal concentration has on shoot herbivores, is by using within-species variation in foliar metal concentration when grown under a standard metal-spiked growth medium, as it eliminates environmentally induced variation (Jhee *et al.* 1999). The use of low and high foliar metal concentration produced by natural genetic variation (Jhee *et al.* 1999), genetic crosses between hyperaccumulators and non-hyperaccumulators (Huitson & Macnair 2003), different ecotypes (Jiang *et al.* 2005; Noret *et al.* 2005) or natural metallicolous population differences (Boyd *et al.* 2002) has been investigated. However these few studies only use hyperaccumulating species. In these studies the herbivores are given a choice between very highly contrasting foliar metal concentrations, and they significantly prefer plants with the lower foliar metal concentration (Jhee *et al.* 1999; Boyd *et al.* 2002; Jiang *et al.* 2005). However, if a higher foliar metal concentration does have a selective advantage, it is likely that this would have been a stepwise procedure, based on small differences within a population and between species. Therefore the use of less contrasting choices, through the use of population differences in metal-accumulating plants is warranted (Boyd & Moar 1999).

3.1.1 Chapter Aims & Null Hypotheses:

Thus to better understand the potential advantage that higher foliar metal concentrations may confer to plants by reducing their palatability, the pot trials in this thesis will use and compare metallicolous populations of the Zn accumulator *R. acetosa*. This chapter uses two novel approaches to investigate the effect of foliar Zn concentration on herbivore preference – the use of elevated foliar metal concentrations produced by a non-Zn hyperaccumulating plant (*R. acetosa*), and the manipulation of the foliar Zn concentration through population differences under controlled conditions, as well as through the soil Zn concentration. To do this, population differences in the foliar Zn concentration must be established first, using controlled conditions, as the differences in foliar Zn concentration seen in the field may be linked to site differences instead. To explore the potential population differences and their effect on palatability, two pot trials were set up independently.

3.1.1.1 Pot Trial 1 - Shoot Differences in Metallicolous *R. acetosa* Populations:

This study compares the shoot parameters of three metallicolous populations Black Rocks (BR), Clough Wood (CW) and Gang Mine (GM) of *R. acetosa* and the preference by the generalist snail *Helix aspersa*, when grown from seed under controlled conditions in a standard moderately contaminated Zn-spiked growth medium of 5000 mg/kg. Any significant differences seen in a controlled growth environment are therefore population differences rather than site differences. Therefore this experiment will be able to test if there is variation in the metal tolerance and palatability of the three populations and how the foliar Zn concentration may affect herbivory at concentrations lower than hyperaccumulating levels.

The null hypotheses for Pot Trial 1 were as follows:

1. There is no significant difference in the foliar Zn concentration, concentration factor or shoot biomass of the BR, CW and GM metalicolous *R. acetosa* populations, when grown at 5000 mg/kg soil Zn concentration.
2. There is no significant difference in the percentage consumed between the three metalicolous populations of *R. acetosa*, in trials using adult and juvenile *H. aspersa*.

3.1.1.2 Pot Trial 2 - Preference Trial Manipulating Foliar Zn Concentrations through Soil Zn Concentration and Population Differences:

This study compares the shoot parameters of two metalicolous populations (CW & GM) of *R. acetosa*, when grown under controlled conditions at contrasting soil Zn concentrations representative of moderately contaminated sites (1500 mg/kg) and highly contaminated sites e.g. bare spoil heaps (45,000 mg/kg). CW and GM were chosen for further palatability trials based on the population differences in foliar Zn concentration and CF found in Pot Trial 1. This study investigated the preference of adult and juvenile *H. aspersa* when presented with two leaves - either comparing the two soil Zn concentrations within a population, or comparing the two populations within a soil Zn treatment. Therefore the foliar Zn concentration of *R. acetosa* was manipulated using the soil Zn concentration, and also by using the population differences found in the previous pot trial. Pot Trial 2's experiment design takes into account not only the magnitude of the contrast in foliar Zn concentration required for the herbivore to be able to distinguish between the food offered to it, but also the impact that growth under and tolerance to the contrasting soil

Zn treatments may have on the chemistry and nutrition quality of *R. acetosa*, which could have an unknown effect on the preference of *H. aspersa*.

The null hypotheses for Pot Trial 2 were as follows:

1. There is no statistically significant difference or interaction in the foliar total Zn concentration, concentration factor, or the shoot biomass of the CW and GM *R. acetosa* populations when grown at either 1500 mg/kg or 45,000 mg/kg soil Zn concentration.
2. There is no statistically significant difference in the preference by adult and juvenile *H. aspersa* for either the CW or GM *R. acetosa* populations grown at either 1500 mg/kg or 45,000 mg/kg soil Zn concentrations.

3.2 Methods:

3.2.1 *Plant Propagation for Both Pot Trials:*

Seeds of the BR, CW and GM *R. acetosa* populations were collected from the respective sites in July 2008 and used for pot trial 1, and also in July 2009 for use in pot trial 2. All plant propagation took place in the University of Sussex's glasshouses, under temperatures of 15- 25 °C, and a 16: 8 hr. (light: dark) photoperiod, using supplementary lighting (Osram SON-T 400 W lamps), and were gently watered from above every day using tap water. For both pot trials, seeds were sown thinly and evenly in damp vermiculite, under supplementary lighting. Two weeks from the emergence of the first seedling, seedlings with a total of three to four leaves and with uniform root length, were randomly transplanted to plug cells filled with damp John Innes seed compost. After a further two weeks, seedlings from both populations with a total five to six leaves and with uniform root length, were then randomly transferred to a soil Zn treatment. The soil Zn treatments consisted of a 15 cm diameter pot with a 6

: 1 silver sand : John Innes No. 2 compost mix, spiked with zinc oxide (ZnO) (Fisher Scientific, Standard Laboratory Grade), homogenously mixed in a cement mixer, to produce the required Zn concentration based on the DW of the growth medium.

For **pot trial 1** (carried out in 2008), eight replicates each of the BR, CW and GM *R. acetosa* populations were grown singly in a pot with a growth medium Zn concentration of 5000 mg/kg (DW). For **pot trial 2** (2009), ten replicates each of the CW and GM *R. acetosa* populations were grown singly in a pot with a growth medium Zn concentration of 1500 or 45,000 mg/kg (DW). Pots were placed in a random layout on the bench and on saucers to prevent to the loss of growth medium (and a subsequent decrease in soil Zn concentration) from watering.

3.2.2 *Herbivore Preference Trials:*

Adult and juvenile *Helix aspersa* were collected from residential areas of Southern England, where molluscicides had not been knowingly applied in the previous year. Therefore snails did not have experience of metalliferous sites or metallicolous plants. Adults were identified by the presence of a 'lip' on the shell, and juveniles by the absence of the 'lip' (Kerney & Cameron 1979). Snails were maintained in glass tanks lined with compost, misted daily and kept under natural lighting at 20 °C. Snails were denied access to food for at least 24 hours, to ensure that their appetites were not sated to allow feeding on the plants. Snails had been previously observed to eat non-metallicolous *Rumex* spp. For both pot trials, the herbivore preference trials were carried out when the plants had been in the soil Zn treatment for six weeks, and used leaves that were not senescing or discoloured in anyway. Each replicate consisted of one snail in a misted plastic tub (volume 750 ml), with using leaves of a uniform

surface area, randomly arranged around the snail. For **pot trial 1**, one leaf from each of the BR, CW and GM populations was placed in the tub. For **pot trial 2**, pairs of leaves consisted of the following combinations: 1) CW 1500 mg/kg vs. CW 45,000 mg/kg, 2) GM 1500 mg/kg vs. GM 45,000 mg/kg, 3) CW 1500 mg/kg vs. GM 1500 mg/kg and 4) CW 45,000 mg/kg vs. GM 45,000 mg/kg. For both trials, the stalk of each leaf was wrapped in moist cotton wool and placed in a labelled micro-centrifuge tube, to prevent moisture loss over the trial period. For **pot trial 1**, after 24hrs. the leaf area removed was estimated by eye (aided by previously recording the dimensions of the whole leaf). For **pot trial 2**, after 72 hrs. the leaf area removed was estimated by eye (aided by previously tracing the whole leaf). For both pot trials, leaves used in the preference trial had their fresh weights recorded, so that the shoot biomass of the whole plant could be deduced, based on the FW:DW ratio of the unused leaves. The original plant was destructively harvested as described below.

3.2.3 *Plant Harvesting Procedure for Both Pot Trials:*

Plants were grown in the soil Zn treatments for six weeks before the shoot material was destructively harvested from the basal node. Shoot material was carefully washed under running tap water to ensure that all traces of ZnO were removed from the surface before chemical analysis of foliar Zn concentration. Plant samples were dried at 60°C for 48 hours before having their shoot dry weight (DW) (g) recorded. Plant samples were ground to a dry, fine powder (<180 µm) using a Fritsch Mini-Pulverisette 23 with stainless steel accessories (Zn-free with negligible contamination from Ni, Cr and Fe), and stored in polythene self-seal bags.

3.2.4 *Foliar Zn Concentration Analysis:*

3.2.4.1 Plant Sample Preparation and Digestion:

Plant samples were prepared and digested as according to the methods presented in chapter 2, section **2.2.6.2.** (*Foliar Zn Concentration Analysis*).

3.2.4.2 Blank Correction, Bias and Precision:

Reagent and matched reagent blank corrections, and the calculation of the bias and precision, were carried out according to the methods and equations presented in chapter 2, section **2.2.6.3.** (*Blank Correction, Bias and Precision*).

3.2.4.3 Flame Atomic Absorption Spectrometry:

F-AAS instrumental parameters, and details of the calibration standards and set-up used, were as described in chapter 2, section **2.2.6.4.** (*Flame Atomic Absorption Spectrometry*).

3.2.5 *Concentration Factor:*

The concentration factor (CF) is defined and calculated as according to chapter 2, section **2.2.7.** (*Concentration Factor*).

3.2.6 *Statistical Analysis:*

Assumptions of equal variances and normal distributions for parametric statistics were tested for using Levene's Test and the Kolmogorov- Smirnov test (Zar 2010). Data were transformed if it did not meet these assumptions, and non-parametric statistics were used if transformation did not meet the requirements of the statistical test. So that the figures could provide a clear

visual representation of the trends, the mean and SE mean was presented for the percentage of leaf consumed data, instead of the median and IQR.

3.2.6.1 Pot Trial 1 - Shoot Differences in Metallicolous *R. acetosa* Populations:

For the *R. acetosa* plants, one-way ANOVAs were used to determine if a) the SQRT-transformed foliar Zn concentration, b) the SQRT-transformed concentration factor and c) shoot biomass (DW) differed between populations. Post-hoc Tukey tests were used to determine which levels were significantly difference from each other when $p < 0.05$. A non-parametric version of the two-way ANOVA called the Scheirer-Ray-Hare (SRH) test was used to determine the effects of 'Plant Population' and 'Snail Age' (fixed factors) and their interaction on the ranked values of percentage leaf area consumed. Post-hoc Tukey tests on the ranked data were used to determine which levels were significantly difference from each other when $p < 0.05$.

3.2.6.2 Pot Trial 2: Preference Trial Comparing Foliar Zn Concentrations through Manipulation of Soil Zn Concentration and Population Identity:

GLMs were used to determine if the a) shoot biomass, b) foliar Zn concentration and c) LN transformed concentration factor of the two *R. acetosa* metallicolous populations differed between the fixed factors 'Soil Zn treatment' and 'Population' and if there was an interaction between these factors. Post-hoc Tukey tests were used to determine which levels were significantly different from each other when $p < 0.05$. Wilcoxon-paired-sample tests (Zar 2010) were used to see if there was a significant preference for a leaf type, using the ranked paired sample differences in percentage of leaf consumed, between the 1500 and 45,000 mg/kg soil Zn treatments for a) the CW population and b) the GM population, for adult and juvenile *H. aspersa*. Wilcoxon-paired-sample tests were also used to see if there was a significant preference for a leaf type, using

the ranked paired sample differences in percentage of leaf consumed, between the CW and GM populations for a) the 1500 mg/kg and b) the 45,000 mg/kg soil Zn treatments for adult and juvenile *H. aspersa*.

3.3 Results:

3.3.1 *Pot Trial 1 - Shoot Differences in Metallicolous R. acetosa Populations:*

3.3.1.1 Precision and Bias of the Foliar Zn Concentration:

The overall precision (at 95 % confidence) was within satisfactory levels at 4.6 % (n = 6), but the overall bias of the reference materials was + 20.0 % (n= 3) respectively. Since this study focuses on between population differences i.e. internal comparisons, the uncertainty in the absolute values obtained should not be of great consequence to the trends seen.

3.3.1.2 Foliar Zn Concentration:

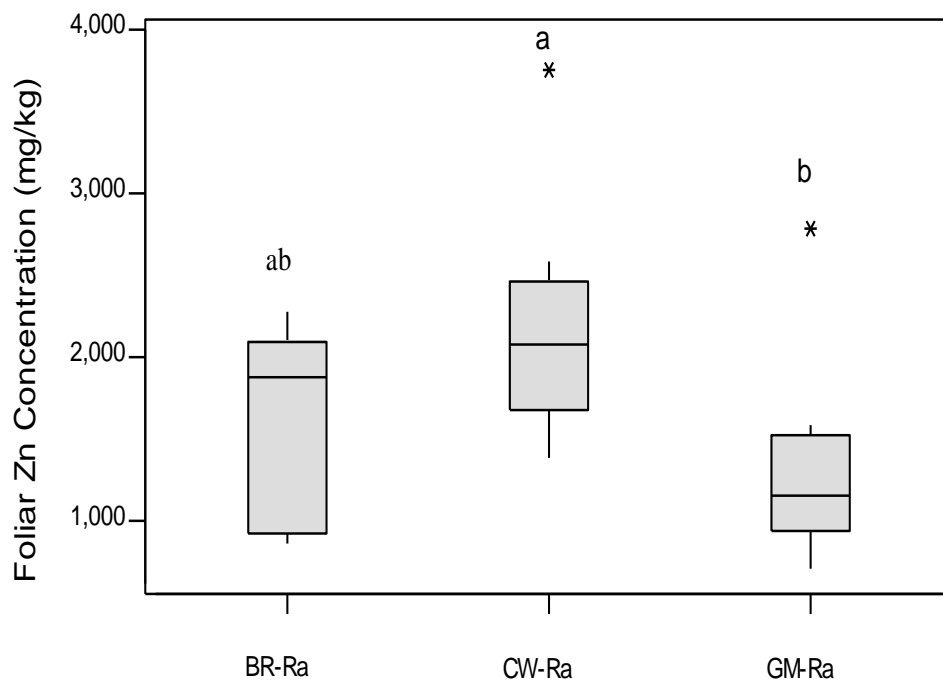


Figure 3- 1: Median and IQR of the foliar Zn concentration (mg/kg) (DW) of three *Rumex acetosa* metalliferous populations grown in a sand-compost mix with a soil Zn concentration of 5,000mg/kg. One way ANOVA on SQRT transformed data: $F = 3.78$, $df = 2$, $p = 0.041$. A post-hoc Tukey test indicates where significant differences lie. '*' indicate outliers.

3.3.1.3 Concentration Factor:

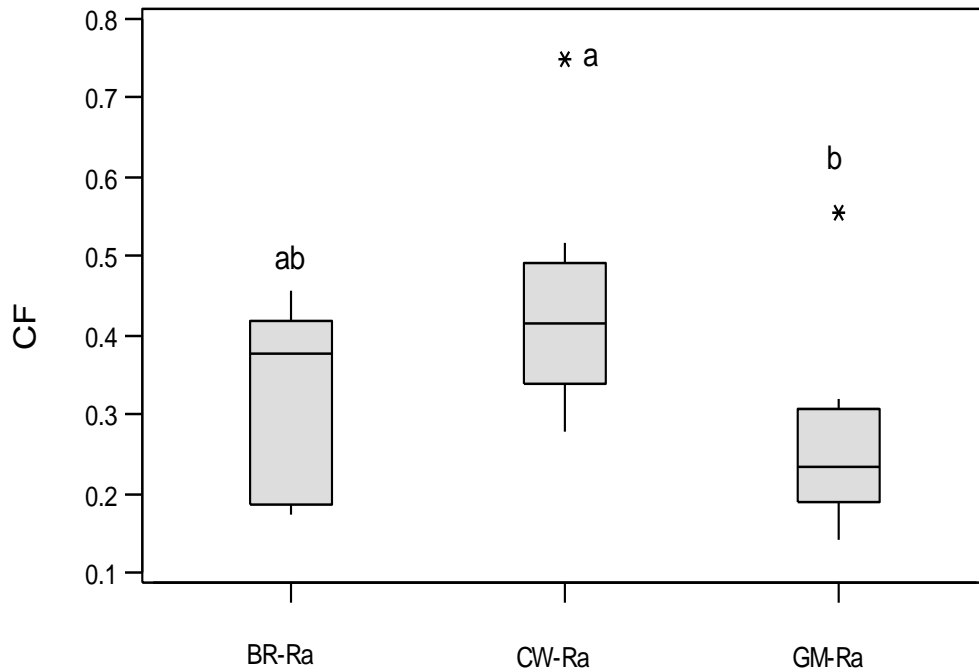


Figure 3- 2: Median and IQR of the concentration factors of three *Rumex acetosa* metalicolous populations grown in a sand-compost mix with a soil Zn concentration of 5,000mg/kg. One way ANOVA on SQRT transformed data: $F = 3.78$, $df = 2$, $p = 0.041$. A post-hoc Tukey test indicates where significant differences lie. '*' indicate outliers.

A significant difference was found in the foliar Zn concentrations and concentration factors of three *R. acetosa* populations grown from seed under greenhouse conditions (**Figure 3- 1 & Figure 3- 2**). The CW population of *R. acetosa* displayed a significantly higher foliar Zn concentration and concentration factor than the GM population when grown at 5,000 mg/kg soil Zn concentration.

3.3.1.4 *Preference by Adult and Juvenile Helix aspersa.*

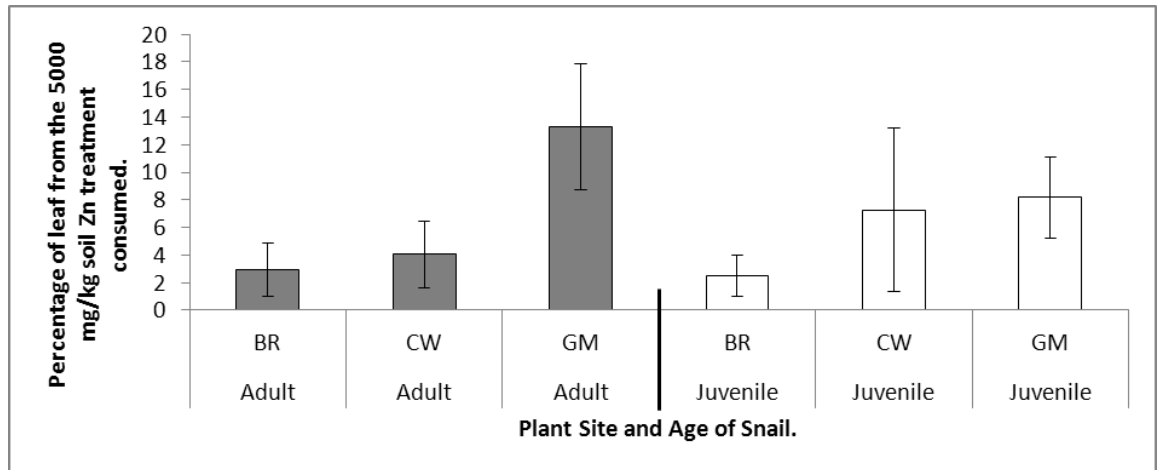


Figure 3- 3: Mean & SE mean of the percentage of excised leaf consumed by adult (grey bars) and juvenile (white bars) *Helix aspersa* when presented with three *Rumex acetosa* metalicolous populations grown in a sand-compost mix with a soil Zn concentration of 5,000mg/kg. The statistical output of a SRH test is displayed in Table 3- 1. Although a significant difference was found for population, post-hoc Tukey pairwise comparisons were not significantly different from each other.

SRH Output for Percentage Leaf Consumption.						
Source	DF	Seq SS	Adj SS	Adj MS	Adj SS/MS total	P value
Snail Age	1	84.0	84.0	84.0	0.27873	0.598
Population	2	2129.0	2129.0	1064.5	7.06441	0.029
Snail Age * Population	2	24.2	24.2	12.1	0.07964	0.961
Error	54	15543.4	15543.4	287.8		
Total	59	17780.5		301.37		

Table 3- 1: Statistical output of the SRH test percentage leaf consumption by adult and juvenile *Helix aspersa* for three metalicolous populations of *Rumex acetosa*. Plants were grown at 5,000 mg/kg soil Zn concentration.

When presented with an excised leaf from each of the three *R. acetosa* populations grown at 5,000 mg/kg soil Zn concentration, there was a significant difference in the percentage consumed of each population and this trend did not differ between adult and juvenile *Helix aspersa*, nor interact with snail age (Figure 3- 3 & Table 3- 1). Adult and juvenile snails consumed the GM population most and the BR population least.

3.3.1.5 Shoot Biomass:

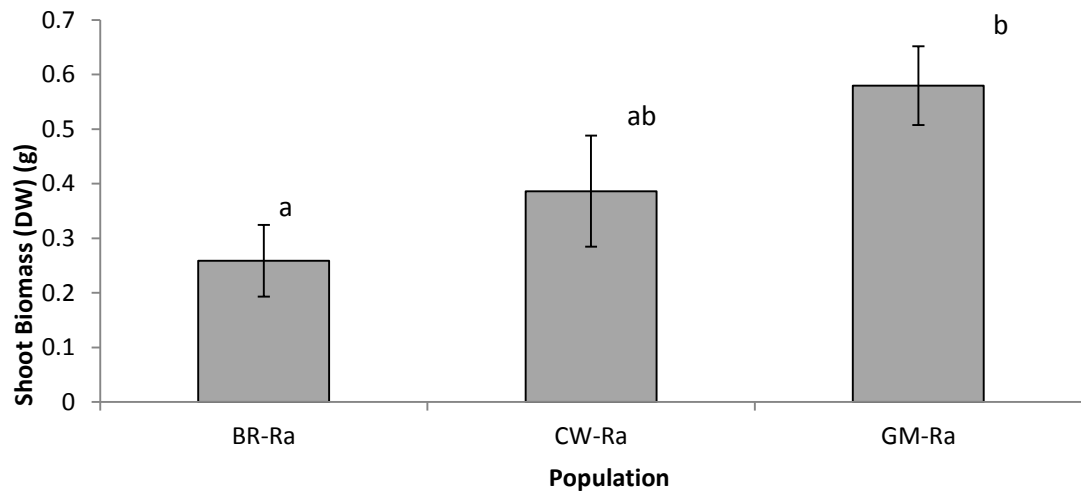


Figure 3- 4: Mean and SE Mean of the shoot biomass DW (g) of three *Rumex acetosa* metalicolous populations grown in a sand-compost mix with a Zn concentration of 5,000mg/kg. One way ANOVA: $F = 3.74$, $df = 2$, $p = 0.042$. A post-hoc Tukey test indicates where significant differences lie. $N = 8$

A significant difference was found in the shoot DW of the three *R. acetosa* populations when grown at 5,000 mg/kg soil Zn concentration (**Figure 3- 4**). The GM population was the largest and the BR population the smallest. The CW and GM population shoot DWs are not significantly different from each other at 5,000 mg/kg soil Zn concentration.

3.3.2 *Pot Trial 2: Preference Trial Comparing Foliar Zinc Concentrations through Manipulation of Soil Zinc Concentration and Population Identity:*

3.3.2.1 *Shoot Biomass:*

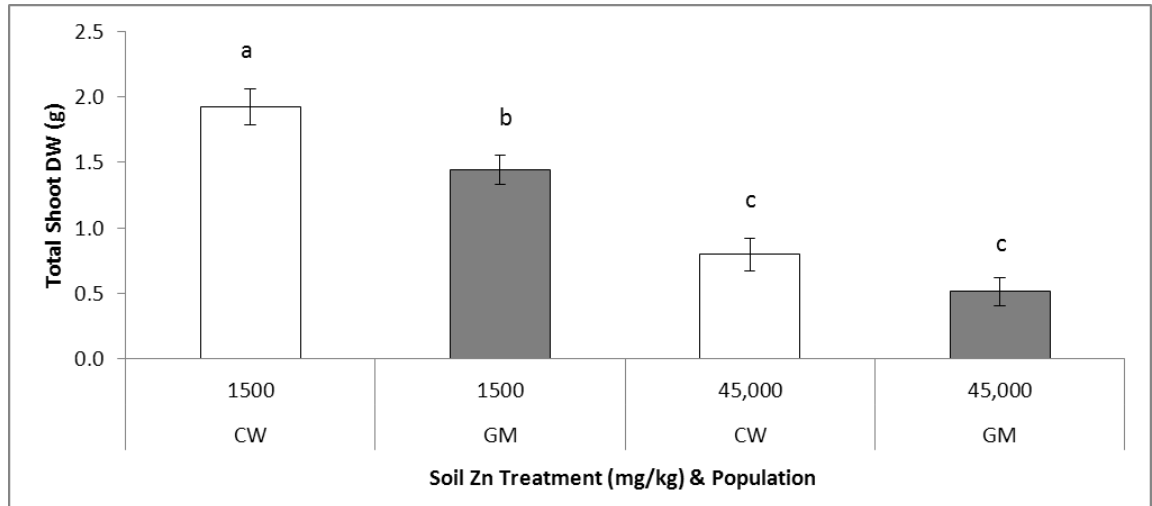


Figure 3- 5: Mean and SE Mean of shoot biomass (DW) (mg/kg) of two metalicolous populations of *Rumex acetosa* (CW and GM), grown in a sand-compost mix with a soil Zn concentration of 1500 and 45,000 mg/kg. The statistical output of a GLM on the data is displayed in Table 3- 2. A post-hoc Tukey test indicates where significant differences lie. N = 10 each.

GLM for Shoot Biomass (DW).						
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Population	1	1.4427	1.4427	1.4427	10.15	0.003
Soil Zn Treatment	1	10.5594	10.5594	10.5594	74.28	<0.001
Population *Soil Zn Treatment	1	0.0946	0.0946	0.0946	0.67	0.420
Error	36	5.1174	5.1174	0.1421		
Total	39					

Table 3- 2: Statistical output of a GLM on shoot biomass (DW), of two metalicolous populations of *Rumex acetosa*, grown in 1500 or 45,000 mg/kg soil Zn treatments.

Both metalicolous populations of *R. acetosa* grown at the 1500 mg/kg soil Zn treatment had a significantly greater shoot biomass than those grown in the 45,000 mg/kg treatment (**Figure 3-5 & Table 3- 2**). In this experiment the CW population had a significantly greater shoot biomass than the GM population

when grown at 1500 mg/kg soil Zn treatment; however this trend was not significant for the 45,000 mg/kg treatment.

3.3.2.2 Precision and Bias of Foliar Zn Concentration:

The overall precision (at 95 % confidence) of the plant samples was within satisfactory levels at 8.4 % (n = 34), and the overall bias of the reference materials was again within satisfactory levels at 6.4% (n = 4).

3.3.2.3 Foliar Zn Concentration:

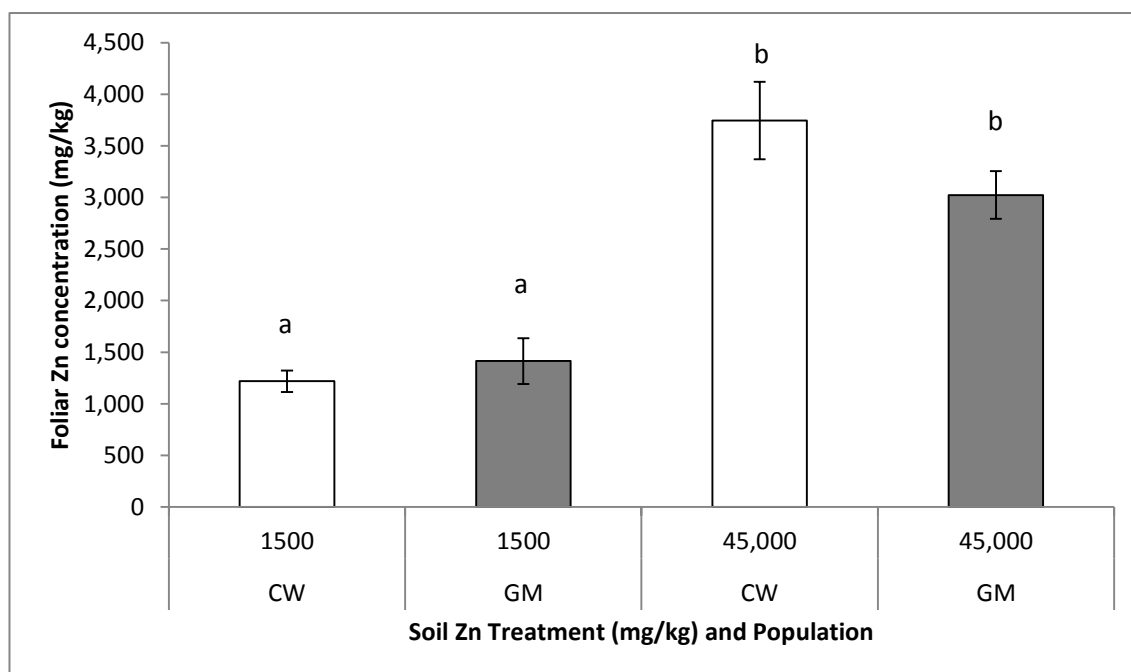


Figure 3- 6: Mean and SE Mean of the foliar Zn concentration (DW) (mg/kg) of two metalicolous populations of *Rumex acetosa* (CW and GM), grown in a sand-compost mix with a soil Zn concentration of 1500 and 45,000 mg/kg. The statistical output of a GLM on the data is displayed in Table 3- 3. A post-hoc Tukey test indicates where significant differences lie. N =10, except GM 45,000 mg/kg where N =8.

	GLM for Foliar Zn Concentration.					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Population	1	1175120	650043	650043	1.05	0.313
Soil Zn Treatment	1	41403930	40205963	40205963	64.89	<0.001
Population *Soil Zn Treatment	1	1973169	1973169	1973169	3.18	0.083
Error	34	21064960	21064960	619558		
Total	37	65617179				

Table 3- 3: Statistical output of a GLM on foliar Zn concentration, of two metalicolous populations of *Rumex acetosa*, grown in 1500 or 45,000 mg/kg soil Zn treatments.

Both metalicolous populations of *R. acetosa* grown at the 45,000 mg/kg soil Zn treatment had a significantly greater foliar Zn concentration than those grown in the 1500 mg/kg treatment (**Figure 3- 6 & Table 3- 3**). The population differences in the foliar Zn concentration seen previously within a soil Zn treatment were not significant in this experiment. However there is a slight population difference, especially for the 45,000 mg/kg treatment.

3.3.2.4 Concentration Factor:

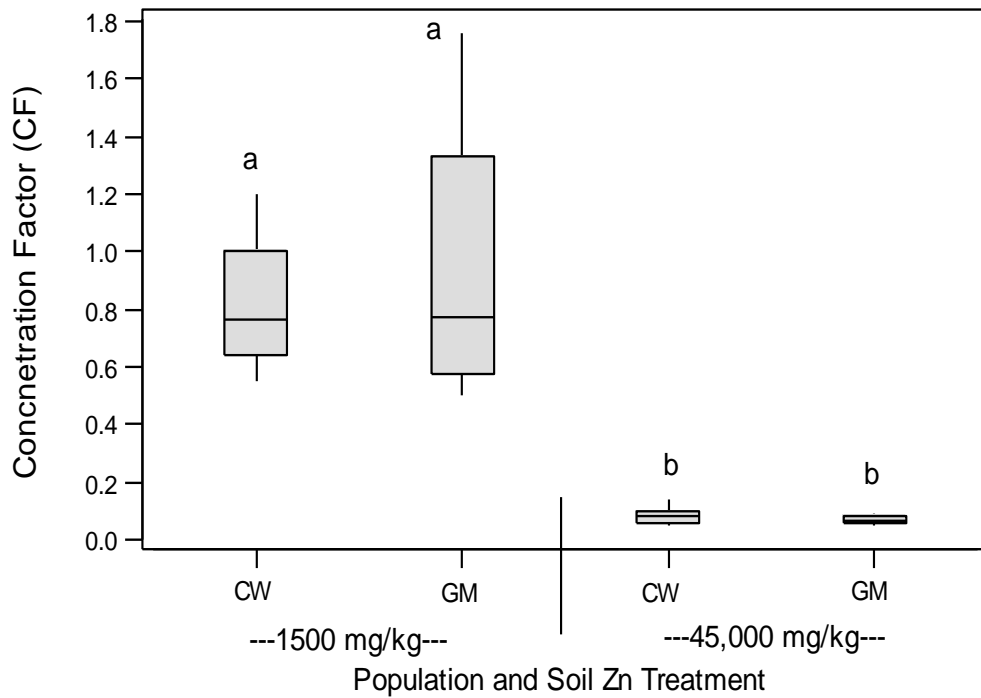


Figure 3- 7: Median and IQR of the concentration factor of two metalliferous populations of *Rumex acetosa* (CW and GM), grown in a sand-compost mix with a soil Zn concentration of 1500 and 45,000 mg/kg. The statistical output of a GLM on the LN transformed data are displayed in Table 3- 4. A post-hoc Tukey test indicates where significant differences lie. N =10, except GM 45,000 mg/kg where N =8.

	GLM output for the LN transformed Concentration Factor.					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Population	1	0.068	0.032	0.032	0.29	0.593
Soil Zn Treatment	1	55.760	55.940	55.940	505.65	<0.001
Population * Soil Zn Treatment	1	0.180	0.180	0.180	1.62	0.211
Error	34	3.761	3.761	0.111		
Total	37	59.769				

Table 3- 4: Statistical output of a GLM on the LN transformed concentration factor, of two metallicolous populations of *Rumex acetosa*, grown in 1500 or 45,000 mg/kg soil Zn treatments.

Both metallicolous populations of *R. acetosa* grown at the 45,000 mg/kg soil Zn treatment had a significantly lower CF than those grown in the 1500 mg/kg

treatment (**Figure 3- 7 & Table 3- 4**). The population differences in the concentration factor seen previously within a soil Zn treatment were not significant in this experiment.

3.3.2.5 Preference Trial – Between Soil Zn Treatments:

Clough Wood:

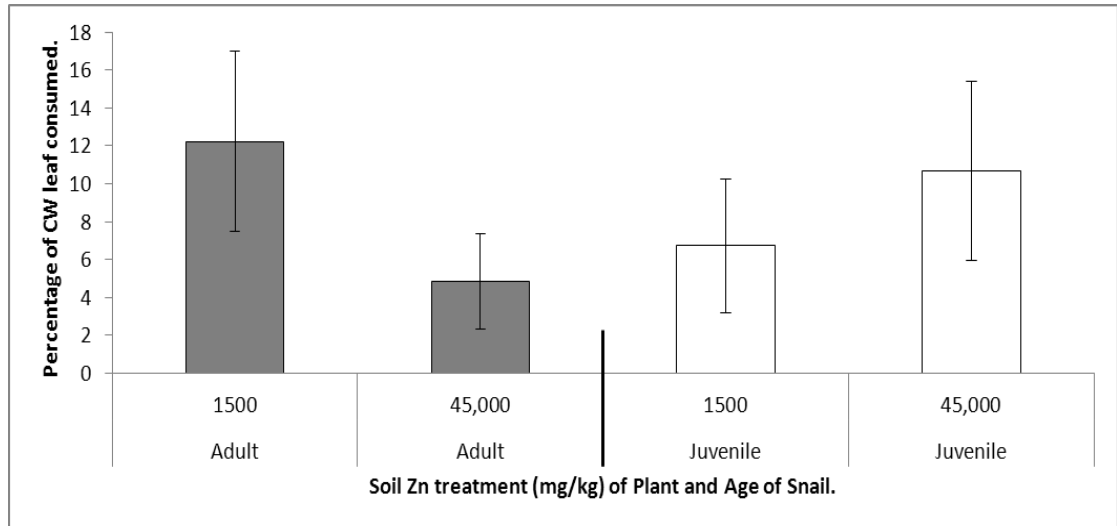


Figure 3- 8: Mean and SE Mean of the percentage consumed of excised CW *Rumex acetosa* leaves, when presented with a leaf from both soil Zn treatments, by adult (n=10) and juvenile (n=10) *Helix aspersa* over a 72 hr. period. Wilcoxon-paired-sample test: Non-significant for adults and juveniles ($T_{0.05, 2}$ tailed, n = 8 and 10 respectively).

When presented with a pair of excised CW *R. acetosa* leaves, one from each soil Zn treatment, there was no significant difference in the percentage consumed between the soil Zn treatments, for both adult and juvenile *H. aspersa*, over a 72 hour time period (**Figure 3- 8**).

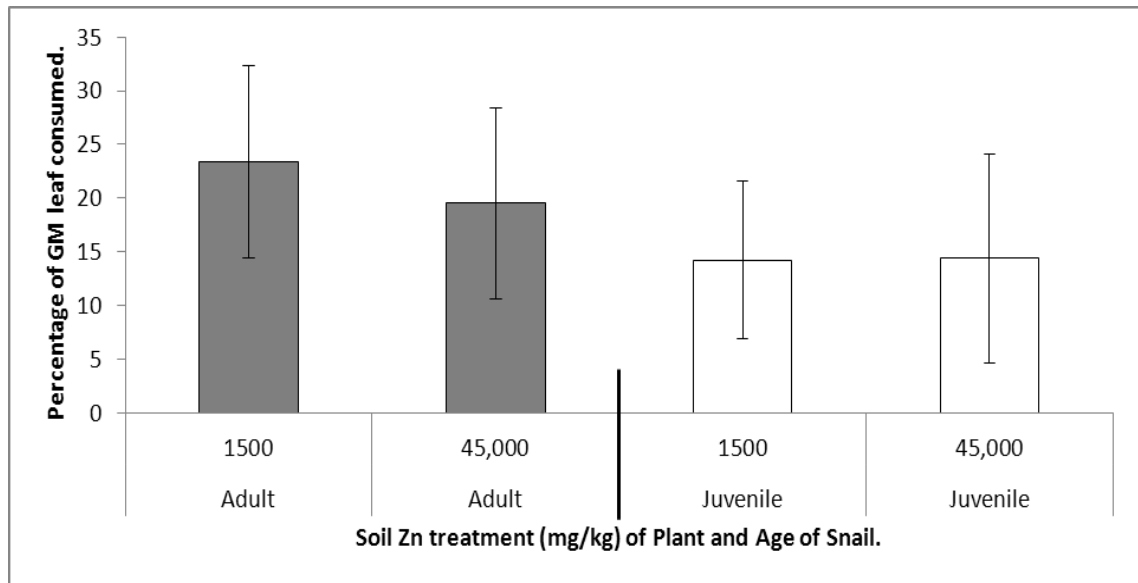
Gang Mine:

Figure 3- 9: Mean and SE Mean of the percentage consumed of excised GM *Rumex acetosa* leaves, when presented with a leaf from both soil Zn treatments, by adult (n=10) and juvenile (n=10) *Helix aspersa* over a 72 hr. period. Wilcoxon-paired-sample test: Non-significant for adults and juveniles ($T_{0.05, 2}$ tailed, n = 10 and 9 respectively).

When presented with a pair of excised GM *R. acetosa* leaves, one from each soil Zn treatment, there was no significant difference in the percentage consumed between the soil Zn treatments, for both adult and juvenile *H. aspersa*, over a 72 hour time period (**Figure 3- 9**).

3.3.2.6 Preference Trial – Between Populations:

1500 mg/kg Soil Zinc Treatment:

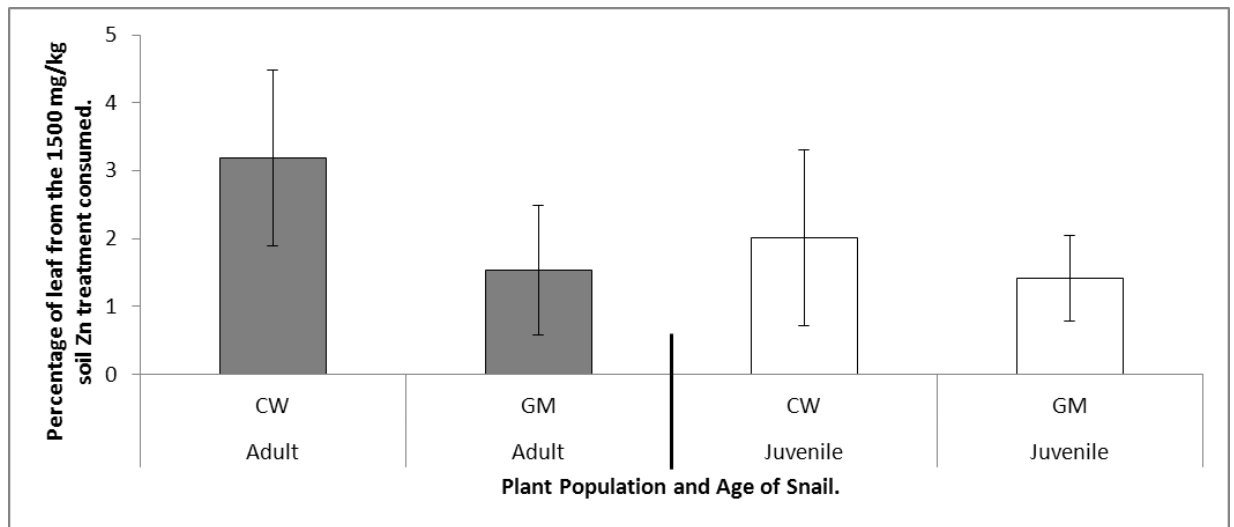


Figure 3- 10: Mean and SE Mean of the percentage consumed of excised *Rumex acetosa* leaves grown in the 1500 mg/kg soil Zn treatment, when presented with a leaf from each of the two populations, by adult (n=10) and juvenile (n=10) *Helix aspersa* over a 72 hr. period. Wilcoxon-paired-sample test: Non-significant for adults and juveniles ($T_{0.05, 2 \text{ tailed}}$, $n = 8$ and 8 respectively).

When presented with a pair of excised *R. acetosa* leaves grown in the 1500 mg/kg soil Zn treatment, one from each population, there was no significant difference in the percentage consumed between the two populations, for both adult and juvenile *H. aspersa*, over a 72 hour time period (**Figure 3- 10**).

45,000 mg/kg Soil Zinc Treatment:

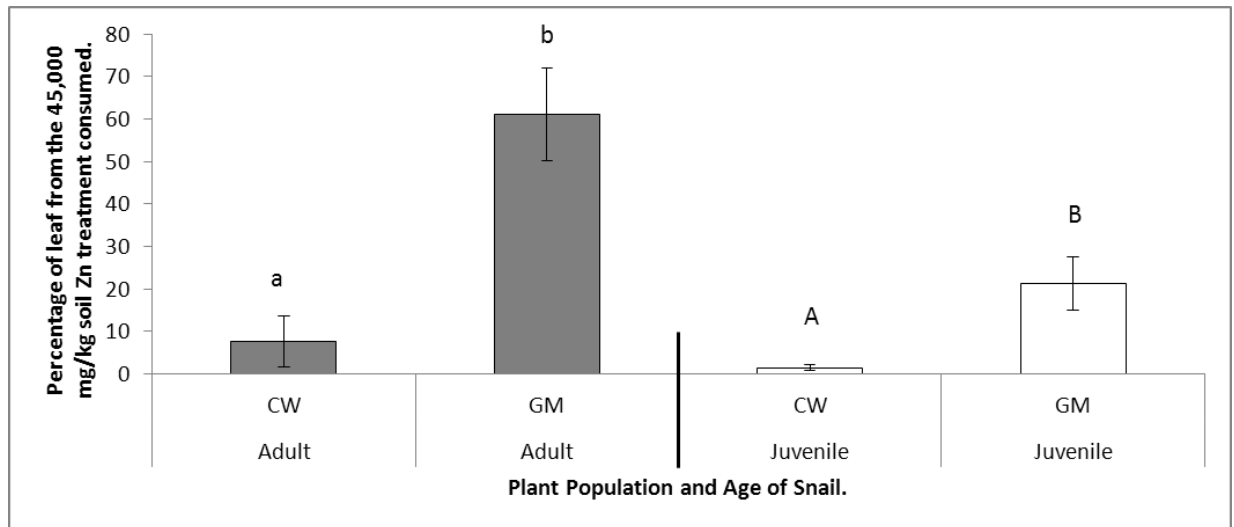


Figure 3- 11: Mean and SE Mean of the percentage consumed of excised *Rumex acetosa* leaves grown in the 45,000 mg/kg soil Zn treatment, when presented with a leaf from each of the two populations, by adult (n=10) and juvenile (n=10) *Helix aspersa* over a 72 hr. period. Wilcoxon-paired-sample test: Significant differences ($p < 0.05$) found for adults and juveniles ($T_{0.05}$, 2 tailed, $n = 8$ and 8 respectively). Separate post-hoc Tukey pairwise comparisons are shown for adults (lowercase letters) and juveniles (uppercase letters).

When presented with a pair of excised *R. acetosa* leaves grown in the 45,000 mg/kg soil Zn treatment, one from each population, there was a significant difference in the percentage consumed between the two populations, for adult and juvenile *H. aspersa*, over a 72 hour time period (**Figure 3- 11**). Excised leaves from GM plants grown at 45,000 mg/kg were significantly preferred over their CW counterparts for both adult and juvenile snails.

3.3.3 *Summary of the Results:*

3.3.3.1 Pot Trial 1 - Shoot Differences in Metallicolous *Rumex acetosa* Populations:

R. acetosa from three metallicolous seed populations (2008), grown at 5000 mg/kg soil Zn concentration, showed population differences in their foliar Zn concentration, concentration factor, shoot biomass and leaf consumption by *H. aspersa*, thus rejecting both null hypotheses.

Growth at a 5000 mg/kg soil Zn concentration produced elevated foliar Zn concentrations of between 700-2500 mg/kg, sometimes reaching 4000 mg/kg. The CW population showed significantly higher foliar Zn concentrations than GM. All populations demonstrated restricted Zn uptake, as the concentration factor (CF) was < 0.5 for all populations. Significant variation was seen in the CF, with the GM population having a significantly lower CF compared to CW. Therefore the GM population restricted the uptake of Zn from the soil into its shoots more so than the CW population under controlled conditions.

When presented with leaves from each metallicolous population grown at 5000 mg/kg soil Zn concentration, consumption by *H. aspersa* over a 24 hr. period significantly differed. No leaves were completely eaten; indeed generally less than 25% of any one leaf was consumed over this period. A significant difference in the percentage consumed of each population was observed, with both adult and juvenile snails consuming the GM population most and the BR population least. This trend did not interact with snail age.

3.3.3.2 Pot Trial 2: Preference Trial Comparing Foliar Zn Concentrations through Manipulation of Soil Zn Concentration and Population Identity:

R. acetosa from two metallicolous seed populations (2009), grown at contrasting soil Zn concentrations, showed variation in their foliar Zn concentration, CF, shoot biomass and leaf consumption by *H. aspersa*, again rejecting both null hypotheses.

Growth in the 45,000 mg/kg soil Zn treatment compared to the 1500 mg/kg treatment affected the shoot biomass of both populations negatively and hence can be classed as an abiotic stress. The CW population had a greater shoot biomass than the GM population for both soil Zn treatments; however this was only significant for the lower soil Zn treatment, suggesting that population differences in shoot biomass are reduced under higher soil Zn concentrations.

The two soil Zn treatments produced significant differences in the foliar Zn concentration. Growth at 1500 mg/kg soil Zn treatment produced elevated foliar Zn concentrations of between 750-2600 mg/kg, and growth in the 45,000 mg/kg soil Zn treatment produced significantly higher foliar Zn concentration of between 2100-6100 mg/kg. However, no significant population differences were seen in the foliar Zn concentration at either soil Zn concentration, although the CW population had a slightly higher foliar Zn concentration in the 45,000 mg/kg soil Zn treatment.

The two soil Zn treatments produced significant differences in the CF. At 1500 mg/kg the CF was approximately 1 for both populations, indicating that passive Zn shoot uptake was occurring. At 45,000 mg/kg the CF was < 0.1 , indicating that *R. acetosa* was strongly restricting Zn uptake into its shoots. However, no

significant population differences were seen in the foliar Zn concentration at either soil Zn concentration.

Plants grown in the 1500 mg/kg soil Zn treatment had a significantly lower foliar Zn concentration than those grown in the 45,000 mg/kg treatment, by a difference of approximately 2000 mg/kg. Hence the soil Zn treatments used successfully produced a wide range of foliar Zn concentrations that were statistically different to provide a contrasting diet choice for *H. aspersa*. For both *R. acetosa* populations, adult *H. aspersa* generally consumed a greater proportion of the leaves from plants grown in the lower soil Zn treatment than those in the higher soil Zn treatment, although this was non-significant and very variable, possibly due to a low sample number. Juveniles showed no preference between plants grown in contrasting soil Zn treatments.

Regarding the between-population differences within the 1500 mg/kg soil Zn treatment, the CW population had a non-significantly lower foliar Zn concentration compared to the GM population, by approximately 200 mg/kg. *H. aspersa* consumed a greater proportion of the CW plants compared to the GM plants, but again this was non-significant and very variable, possibly due to a low sample number. This trend was seen for both adult and juvenile *H. aspersa*.

For the 45,000 mg/kg soil Zn treatment, the GM population instead had a non-significantly lower foliar Zn concentration compared to the CW population, by approximately 700 mg/kg. In this trial only, *H. aspersa* consumed a significantly greater proportion of the GM plants compared to the CW plants. Again this trend was seen for both adult and juvenile *H. aspersa*.

3.4 Discussion:

Metal tolerance was demonstrated in *R. acetosa* when grown from seed from metalicolous populations, due to its ability to grow on moderately and highly contaminated soils. Growth under highly contaminated soil Zn concentrations compared to moderately contaminated ones negatively affected the shoot biomass of both populations and hence can be classed as an abiotic stress (Mathys 1977). Additionally, population biomass differences were seen between the moderately and highly Zn contaminated soils in pot trial 2. However this was only significant for the lower soil Zn treatment, suggesting that population differences in shoot biomass are reduced under higher soil Zn concentrations. Both pot trials also demonstrated significant differences between the metalicolous populations, under controlled conditions, suggesting that differences in the metal tolerance in these populations could be a heritable trait, although the genetic confirmation of this is required, which is outside the scope of this thesis.

In both pot trials, foliar Zn concentrations of 1000 - 4000 mg/kg were produced, in moderate and highly Zn contaminated growth mediums. These levels were similar to other metalicolous populations/ accessions of *R. acetosa* grown in highly contaminated soils (Barrutia *et al.* 2009; Epelde *et al.* 2010), and are elevated compared to other pseudometallophytes on metalliferous soils, thus *R. acetosa* foliar Zn concentration can be classed as 'accumulating' (Reeves & Baker 2000). When grown at 1500 mg/kg soil Zn concentration, both metalicolous populations displayed a CF of approximately 1, thus their metal tolerance mechanism can be classed as an 'Indicator', as shoot Zn uptake is neither being restricted nor increased (Baker 1981). However when grown at 5000 and 45,000 mg/kg, the concentration was < 0.5 and < 0.1 respectively, and thus *R. acetosa* is strongly restricting the movement of Zn from the soil into the shoots (Baker

1981). The dependence of this trait on the soil Zn concentration agrees with another study using *R. acetosa* (Epelde *et al.* 2010) , and those using other metallicolous populations of non-hyperaccumulators such as *Silene maritima* (Baker 1978; Baker 1981).

Pot trial 1 demonstrated that the CW population had a significantly greater foliar Zn concentration than GM (by approximately 1000 mg/kg), and that in a preference trial the GM population was consumed twice as much as the BR and CW populations by adult snails. Juveniles also showed a slight preference from GM over the other two populations. Therefore when grown under controlled conditions, at 5000 mg/kg soil Zn concentration, the relatively higher palatability of the GM population could be directly affected by its relatively lower foliar Zn concentration, or indirectly affected by the influence of foliar Zn concentration on other plant defences and nutritional status.

By using the contrasting CW and GM populations again in pot trial 2, grown under 1500 and 45,000 mg/kg soil Zn concentrations, the foliar Zn concentration of the plants was successfully manipulated through the soil Zn concentration to produce a highly significantly contrasting choice. The foliar Zn concentration was also manipulated through Zn shoot uptake population differences, although to a lesser degree in pot trial 2 compared to those previously seen in pot trial 1. Therefore this experiment design takes into account the magnitude of the contrast in foliar Zn concentration required for the herbivore to be able to distinguish between the choices offered, and the impact that growth under, and tolerance to, the contrasting soil Zn treatments may have on the chemistry and nutrition quality of *R. acetosa*. Both of these factors could have an unknown effect on the preference of *H. aspersa*. In contrast to other studies investigating the EDH in hyperaccumulators through soil metal manipulation with chewing herbivores (Boyd & Martens 1994; Martens & Boyd 1994; Pollard & Baker 1997;

Behmer *et al.* 2005), the increased foliar Zn concentrations in the *R. acetosa* produced from highly contaminated soil were not the major cause of *H. aspersa* deterrence. Instead the population identity when grown at highly contaminated soils produced the most significant difference in palatability. The GM population when grown at 45,000 mg/kg had a significantly higher palatability than the CW population, differing by as much as 50 % in adults and 20 % in juveniles. However this population difference was not seen in the 1500 mg/kg soil Zn treatment, as the percentage eaten of the two populations was very similar, differing by 1- 2 % for both adults and juveniles. Additionally the increase in a population's foliar Zn concentration through growth in the higher soil Zn treatment did not produce a significant difference in palatability as consumption differences were about 10 % for both adults and juveniles. Hence this experiment indicates that the magnitude of the foliar Zn concentration difference used and the deterrence effect are not simply related; as it seems that metallicolous population differences in palatability are more exaggerated at highly contaminated soil Zn concentrations. As a non-significant difference in palatability between soil Zn treatments was found for both *R. acetosa* populations, it seems that within a population, potential changes in the palatability (through possible changes in the plant physiology), produced by the soil Zn concentration, either do not occur or are not detected by adult and juvenile *H. aspersa*. However when grown at highly contaminated soil Zn concentrations, a significant difference in palatability was found, suggesting that the two populations respond differently to growth under 45,000 mg/kg soil Zn concentration. Pot trial 1 also showed significant differences in palatability between populations when grown at 5000 mg/kg, suggesting that population differences in palatability occur at soil Zn concentrations of ≥ 5000 mg/kg. Although population differences in palatability may become more apparent to *H. aspersa* when plants are grown in soil Zn concentrations of ≥ 5000 mg/kg, i.e. when metallicolous population of *R. acetosa* are restricting the shoot Zn uptake

more, further research is required to investigate the physiological interactions between metal tolerance mechanisms, foliar metal concentrations, organic defence compounds and nutritional quality. This highlights the need of the EDH to be further investigated from a combined ecological and physiological perspective. For instance, Huitson & Macnair (2003) crossed the Zn hyperaccumulator *Arabidopsis halleri* with the non-hyperaccumulator *A. petraea*, to produce genetic-based variation in the foliar Zn concentration of the F₂ crosses. *H. aspersa* preferred the lower foliar Zn concentration F₂ plants over *A. halleri*, probably due to the large differences in the foliar Zn concentration presented, a method which usually provides support for the EDH in hyperaccumulating plants (Huitson & Macnair 2003). However, *H. aspersa* did not discriminate between F₂ plants by their foliar Zn concentration but by the growth medium Zn concentration. This suggests that *H. aspersa* metal preference is limited by its ability to distinguish between the reduced range of foliar metal concentrations presented to it using the F₂ plants, and that the growth medium concentration influenced the palatability of the plants, although the nutrition and organic defence compounds were not measured (Huitson & Macnair 2003). Hence the soil metal concentration may influence the physiology and hence palatability of plants in both their inorganic and organic composition.

It has been suggested that a 'trade-off' may exist between elemental and organic defence levels in metallicolous plants compared to non-metallicolous plants (Martens & Boyd 1994; Boyd 1998; Noret *et al.* 2005). The 'trade-off' theory proposes that the elemental defence strategy may be more dominant in metallicolous populations and metal-hyperaccumulating species due to the increase in shoot metal concentrations and that this is linked to a subsequent decrease in the dominance of organic defences (Boyd 1998). The advantage of metals over organic defences has been theorised to include a lower production

cost as they are taken up from the soil, and the metal-complexing molecules, such as citrate and malic acid, have a smaller molecular weight, and hence a low metabolic cost associated with them (Martens & Boyd 1994; Boyd 1998). Additionally, the rarity of metal hyperaccumulating plants (Boyd 1998), potentially makes highly elevated foliar metal concentrations a novel defence strategy, and is less likely to have evolved metal-tolerant herbivores as elemental defence cannot be circumvented by chemical degradation (Boyd 1998). However, studies of specialist, metal-tolerant herbivores and their detoxification mechanisms are limited: Schwartz & Wall (2001) discovered *Melanotrachus boydi* (Heteroptera: Miridae) on the Ni hyperaccumulator *Streptanthus polygaloides*, found on serpentine soils in Sierra Nevada. *M. boydi* was associated with all populations of *S. polygaloides* surveyed and contained elevated Ni concentrations, ten-fold that of normal animal Ni concentrations (Boyd & Wall 2001). Although the mechanism behind Ni-tolerance in this system remains undetermined, more recently an intricate Se detoxification system has been reported by Freeman *et al.* (2006). The Se-hyperaccumulating plant *Stanleya pinnata* (Brassicaceae) was found to be attacked by a Se-tolerant variety of the diamond-backed moth *Plutella xylostella* Stanleyi, and parasitizing the Se-tolerant moth was the wasp *Diadegma insulare*. Non-tolerant plants grown in the presence of Se assimilate Se as selenocysteine (SeCys) with toxic consequences, as SeCys can non-specifically replace cysteine, leading to protein misfolding. However, Freeman *et al.* (2006) determined that the Se-tolerant forms of the plant, moth and wasp could all detoxify Se through accumulation of methylselenocysteine (MeSeCys) instead of SeCys. Feeding trials additionally demonstrated the superior performance of *P. xylostella* Stanleyi on Se-enriched leaves compared to non-tolerant herbivores. Therefore Se tolerance and accumulation through the MeSeCys pathway rather than Se exclusion seems to be a unifying physiological Se-tolerance mechanism at all three trophic levels. Intriguingly, although Se-rich leaves were rejected by non-tolerant herbivores

in feeding and oviposition trials, *P. xylostella* Stanleyi did not distinguish between low and high Se leaves in these trials. This suggests a potential selective advantage to Se tolerance coupled with losing Se aversion, due to the increased reproductive success of Se-tolerant individuals ovipositing on Se hyperaccumulators because of reduced competition and predation (Freeman *et al.* 2006). However, the selective advantage of Se detection ability in Se specialist herbivores and preference for Se-hyperaccumulators, and the effects of Se concentration on other plant palatability aspects should be considered in future work.

The interaction between elemental and organic defences remains complex. A few studies indicate that a trade-off between elemental and organic defences occurs at the species and ecotype level. Davis & Boyd (2000) found that a non-Ni-hyperaccumulating plant species had significantly higher glucosinolate concentrations compared to a Ni-hyperaccumulator. However this appears to be a constitutive difference between the two species as the soil Ni concentration did not interact with the glucosinolate concentration in either species. Additionally clipping and herbivore treatments significantly induced glucosinolates in the hyperaccumulating species, but Ni concentration was not altered (Davis & Boyd 2000). Tolrà *et al.* (2001) found that increasing the growth medium Zn concentration decreased total glucosinolates in *T. caerulea* shoots, especially for aromatic glucosinolates. However the concentration of total glucosinolates, increased in the roots with increasing root Zn concentration. The compound sinalbin was found to decrease in the shoots with increasing foliar Zn concentration, but to increase in the roots with increasing root Zn concentration. They suggested that as the concentration of certain shoot glucosinolates decreased with increasing shoot Zn concentration, a trade-off between elemental and organic defences could occur. Using *H. aspersa*, Noret *et al.* (2005) investigated the influence of foliar Zn, glucosinolate and nitrogen

concentrations, and dry matter content on metallicolous and non-metallicolous ecotypes of *T. caerulescens* grown under contrasting soil Zn treatments. In the majority of the trials, leaves from the metallicolous ecotype were eaten more than the non-metallicolous ecotype, thus disputing the EDH. Additionally low-glucosinolate leaves were generally preferred and metallicolous populations generally had lower glucosinolate concentrations, supporting the trade-off hypothesis when comparing metallicolous plants with non-metallicolous. However, this preference was not demonstrated consistently for all trials and none of the parameters could unanimously explain the difference in palatability of *T. caerulescens*. Therefore the effectiveness of elemental defences compared to organic defences remains to be investigated.

As all plant species have a vast array of organic defence compounds, rather than a 'trade-off', elemental and organic defence compounds may interact additively or synergistically in the 'Joint Effects Theory' (Boyd 2007). This interaction with organic defences could allow even low concentrations of metals or organic defences to deter herbivory and thus reduce the cost of plant defence (Boyd 2007). However, only one study has investigated this interaction so far. Using artificial diets, Jhee *et al.* (2006) demonstrated that although single element concentrations (Ni, Zn, Cd and Pb) were toxic to *Plutella xylostella*, certain metal-metal and metal-organic compounds in combination were significantly more toxic. When Zn was present in the metal-metal combinations, it enhanced the toxic effect of the second metal in an additive manner. When Ni was present in the metal-organic defence compound combinations, it enhanced the organic compound's toxic effect. Therefore this interaction of metals with organic defence compounds suggests that low or 'accumulator' levels of metals influence plant palatability and may reduce the cost incurred in producing organic defences.

In *R. acetosa* the organic acid oxalate has both metal tolerance and herbivore defensive roles. Therefore oxalate may interact with the foliar Zn concentration and affect plant palatability. Plant organic acids are involved in Zn tolerance due to their chelation properties (Marschner 1995) and additionally as anti-herbivore defence compounds (Harborne 1997). Mathys (1977) found that metal- resistance populations of *R. acetosa* and *Silene cucubalus*, were found to have higher levels of malate and to a lesser extent, oxalate, compared to Zn-sensitive populations, when grown in Zn-amended solution. Malate was suggested to be the main complexing agent in the cell plasma and transporter to the cell vacuole, where oxalate then binds to the Zn (Mathys 1977). Jáuregui-Zúñiga (2005) found an interaction between calcium oxalate crystal density and Zn and Pb concentration in beans (*Phaseolus vulgaris*). Plants grown in either Pb or Zn amended solution contained significantly fewer calcium oxalate crystals, than those grown in the metal-control treatments. The presence of the metals decreased the crystal density even under high calcium concentrations. Shoot organic acids are also affected by the presence of other metals. In *R. acetosa* Tolrà *et al.* (2005) found that exposure to Al at non-toxic concentrations produced significant changes in the root and shoot concentration and composition of phenolics and organic acids. In the shoots, Al supply caused a significant decrease in the concentration of the shoot organic acids oxalate, malate and citrate, and also in calcium. Oxalate also has defensive anti-herbivore properties for phloem feeders when in the soluble form (Yoshihara *et al.* 1980; Libert & Franceschi 1987) and in the insoluble form of calcium oxalate crystals for chewing herbivores (Ward, Spiegel & Saltz 1997; Hudgins, Krekling & Franceschi 2003; Korth *et al.* 2006). Thus the presence of shoot organic acids and foliar Zn concentration may have dual and interacting roles in metal tolerance and herbivore defence in metal-tolerant populations of *R. acetosa*.

Further work to untangle the primary factors in the palatability and metal tolerance mechanisms of metalliferous *R. acetosa* populations should include qualitative and quantitative metabolic analysis of the shoot organic acids, specifically malate, citrate and oxalate (Mathys 1977), and the phenolic acids ferulic acid, catechol, catechin and rutin (Tolrà *et al.* 2005). Analysis of the metabolomic differences produced by growth in low and high soil Zn concentrations of non-metalliferous and metalliferous populations would allow further understanding into the influence of metal toxicity and tolerance on plant physiology and population differences within this. Further feeding trials utilizing artificial diets spiked with varying concentrations of Zn and secondary metabolites, and combinations of these to investigate potential joint effects, could then offer further insight into the specific effects of metal tolerance on plant palatability and the relative importance of foliar Zn concentration and secondary metabolites on *R. acetosa* palatability to herbivores.

3.5 Conclusion:

In the current study, metalicolous populations and moderately and highly contaminated soil Zn concentrations were used to manipulate the foliar Zn concentration in order to test the EDH. According to the function of oxalate and organic acids in *R. acetosa* described above, an increase in soil Zn concentration could elicit a change in the shoot organic compounds. However if the soil Zn concentration did produce differences in palatability, these within-population differences were not detectable or did not affect the preference of *H. aspersa*. This was in contrast to the significant effect of growth medium found by Huitson & Macnair (2003) on *H. aspersa* preference of *Arabidopsis* F₂ crosses. As *H. aspersa* did show a population preference at 45,000 mg/kg, this indicates that some aspect of *R. acetosa* palatability is detectable by *H. aspersa*, but only between populations grown under highly contaminated soils. Therefore it

seems that the plant's response to growth in highly contaminated soil Zn concentrations could differ between metallicolous populations, and that this more strongly influences the palatability of the leaves to *H. aspersa*. Further work investigating the qualitative and quantitative changes in the secondary metabolites of *R. acetosa* in response to metal tolerance and population differences, and the effects this may have on plant palatability is suggested.

Chapter 4: The Effect of Inter- and Intra-Population Pairwise Competition on the Competitive Ability and Foliar Zinc Concentration of two Metallicolous Populations of *Rumex acetosa*, grown at Contrasting Soil Zinc Concentrations.

4.1 Introduction:

In view of the Elemental Defence Hypothesis (EDH), **Chapter 3** of this thesis investigated the influence of foliar zinc (Zn) concentration on the preference of the herbivore *Helix aspersa* through differences in metallicolous *Rumex acetosa* populations and soil Zn concentration. Increasing the soil Zn concentration of the growth medium resulted in a decrease in shoot biomass, thus the productivity of even metallicolous populations is reduced under Zn stress. Although the soil Zn treatment also significantly affected the foliar Zn concentration of both populations, *H. aspersa* showed no preference between soil Zn concentrations. A preference was seen between the populations grown at the highest soil Zn concentration, but not at the lowest. Therefore the response to growth in highly contaminated soils differs between metallicolous populations of *R. acetosa*, and this population difference potentially influences the palatability of leaves to *H. aspersa*. Investigation of how these metallicolous population differences affect *R. acetosa* in another ecologically important interaction – plant competition for resources – is now presented in this chapter.

Metallicolous plant populations are subjected to a harsh abiotic environment, which has a low water and nutrient supply and high concentrations of toxic metals (Ye *et al.* 2002). Physiological adaptive mechanisms are required to counteract the toxic effects of elevated metal ions in the soil e.g. the production of metal-binding metabolites, transportation and compartmentalisation in the vacuole (Schat, Llugany & Bernhard 2000). In *R. acetosa*, a study comparing two

accessions from a contaminated and uncontaminated site, grown under a high Zn, Pb and Cd hydroponic solution, demonstrated severe phytotoxic physiological effects in the uncontaminated site accession, namely through the impairment of stomatal function (Barrutia *et al.* 2010). However, these physiological adaptations to metal toxicity potentially require additional energy, resulting in a metabolic cost to the plant (Wilson 1988; Punz & Sieghardt 1993; Ernst 2006; Maestri *et al.* 2010). Thus metabolic costs may result in survival in metal-contaminated soil at the expense of growth and reproductive output, i.e. a trade-off between metal tolerance and fitness (Baker 1987; Ernst, Schat & Verkleij 1990; Wu 1990; Harper, Smith & Macnair 1997a; Kazakou *et al.* 2008; Maestri *et al.* 2010). Support for a trade-off between metal tolerance and growth in non-hyperaccumulating metal-tolerant plants has also been demonstrated by shift in the biomass allocation patterns from the shoots to the roots under increasing soil metal concentration (Audet & Charest 2008). Additionally, Wilson (1988) demonstrated in *Agrostis capillaris* genotypes and populations from mining sites and normal soils, a relatively lower relative growth rate (RGR) in the metallicolous plants which was correlated with a greater Cu tolerance, and to a lesser extent, Pb tolerance. Other studies have also inferred a potential cost to metal tolerance in non-hyperaccumulating plant species (Jowett 1964; Hickey & McNeilly 1975; Brookes, Collins & Thurman 1981; Cox & Hutchinson 1981), but all of these studies have compared metal-tolerate and non-tolerate populations. Wilson (1988) found significant variation in both metal tolerance and productivity within the populations and genotypes used, indicating that although a trade-between metal tolerance and productivity can be seen, the influence of other environmental factors will also influence this relationship.

As metalliferous sites are additionally characterised by a low water and nutrient supply (Ye *et al.* 2002), thus resulting in lower productivity, additional

physiological adaptations are required (Kazakou *et al.* 2008). These adaptations to infertile soils may be genetically independent of the metal tolerance traits (Ernst 2006). For instance, the cost incurred by growth in contaminated soils and its associated metal tolerance adaptive mechanisms, may be alleviated by increased nutrient supply. Barrutia *et al.* (2009) investigated the effect of fertilisation on the growth and Zn uptake of metallicolous and non-metallicolous populations of *R. acetosa*. Fertilisation stimulated the growth of both populations under a range of soil Zn concentrations of approximately 6700 – 20,500 mg/kg, the metallicolous population more-so than the non-metallicolous. Metal toxicity was the limiting factor of growth, as the fertilization treatment did not raise biomass levels to that of the metal-free soil treatment. Fertilisation was shown to improve the phytoextraction potential (i.e. $\mu\text{g metal plant}^{-1} \text{ month}^{-1}$) of metallicolous *R. acetosa* plants. Hence soil and foliar metal toxicity and tolerance can be ameliorated and increased by increased nutrient supply (Smith & Bradshaw 1970; Smith & Bradshaw 1979; Meerts & Van Isacker 1997; Tordoff, Baker & Willis 2000). Superior metal tolerance and growth on mining sites is therefore potentially dependent upon the ability of an individual to capture resources for sustained growth and metal tolerance, in metalliferous soils.

The efficiency of capturing resources which are in limited supply can be investigated using plant competition experiments. In this thesis competition is defined as in Keddy (1989) pg. 2 – “the negative effects which one organism has upon another by consuming , or controlling access to, a resource that is limited in availability”. Resources in this sense, lead to increased growth rates when their availability is increased, such as nutrients, water and light (Keddy 1989). Hence by limiting the availability of resources and implementing competition for these resources in pot experiments, we can investigate the efficiency by which a plant can capture resources for growth, i.e. its competitive ability

(measured by using the shoot biomass). Increased growth and reproduction when under competition demonstrates increased plant fitness. Thus there is a potential trade-off between survival in metal-contaminated soil and competitive ability as well.

The use of competitive ability to measure the tolerance and resource capture ability of populations in their native environmental conditions and contrasting ones has been demonstrated for several edaphic (soil-based) characteristics e.g. soil nutrient status (Shontz & Shontz 1972), and soil pH (Snaydon 1962; Snaydon 1971). Studies investigating metal tolerance have generally compared metallicolous with non-metallicolous populations, growing in non-metalliferous soils, for Cu tolerance (McNeilly 1968; Nicholls & McNeilly 1985), serpentine soils (Higgins & Mack 1987) and Zn/Pb tolerance (Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975). Non-metallicolous populations had a superior competitive ability compared to metal tolerant populations when grown in non-metalliferous soils, not only to their larger absolute biomass, but additionally their relatively significantly larger biomass when grown under inter-population competition (Cook, Lefèbvre & McNeilly 1972). Hickey and McNeilly (1975) additionally demonstrated that metal-tolerant populations had a lower fitness (measured by biomass and mortality) compared to non-metallicolous populations when grown in inter-population competition surrounded by *Lolium perenne*. Therefore the metal tolerance trait has a possible fitness cost associated to it, as seen by the reduction in biomass of the tolerant populations when grown under inter-population competition on non-metalliferous soils, compared to the non-metallicolous population, although this difference was dependent upon the species investigated (Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975; Wilson 1988).

The apparent trade-off between metal tolerance and competitive ability is not conclusive however, as the comparison between metallicolous and non-metallicolous populations potentially allows other adaptations to their contrasting environments to affect the population differences seen (Higgins & Mack 1987; Wilson 1988; Ernst 2006). For instance, the less toxic and more fertile soil status of non-metalliferous habitats produces a less stressful environment in terms of the abiotic conditions. However the greater influence of neighbouring plants on the availability of resources, means that these non-metallicolous populations are more adapted to and tolerant of plant competition instead for resources (Keddy 1989; Grime 2002).

4.1.1 *Chapter Aim:*

By comparing the metallicolous populations used in pot trial 2 of **Chapter 3**, the potential cost to metal tolerance can be investigated by measuring the competitive ability of individuals to capture resources for optimum growth, when grown in metal-rich soils. Rather than using populations from highly contrasting environments, the comparison of metallicolous populations allows for potential confounding influences to be reduced. This study therefore investigates the impact of intra- and inter-population pairwise competition on the shoot biomass, relative competitive intensity (RCI), and foliar Zn concentration of two metal-tolerant populations of *R. acetosa* (CW and GM), and how these impacts differ between moderately and highly contaminated soil Zn concentrations. The CW and GM populations were chosen for further investigation based on the population differences seen in foliar Zn concentration and CF in **Chapter 3**, to investigate how these population differences in shoot zinc concentration affect their competitive ability.

4.1.2 *Null Hypothesis:*

There is no statistically significant difference in the shoot biomass, foliar Zn concentration or RCI, between the CW or GM *R. acetosa* populations, when grown at either 1500 mg/kg or 45,000 mg/kg soil Zn concentrations and when grown under intra- or inter-population competition.

4.2 Methods:

4.2.1 *Experimental Design:*

The experimental design consisted of three treatments as fixed factors – soil Zn treatment, competition type and population identity. There were two levels for each factor: soil Zn treatment (1500 mg/kg and 45,000 mg/kg), competition type (inter- and intra-population competition) and population identity (CW and GM). Pots were randomly arranged in blocks, consisting of one pot per treatment 3-way combination. 20 blocks were used and were designated as a random factor. Therefore a fully randomised, 3 × 2 fully factorial block design was used. Within the competition treatment, a pairwise competition design was used based on the de Wit replacement series (Firbank & Watkinson 1990). The total number of plants in each pot was two, therefore intra-population competition consisted of two plants from one population per pot, and inter-population competition consisted of one plant from each population. In the latter, the position of each population was randomly assigned to the left or right side of the pot in advance to avoid potential positional bias. To ensure statistical independence of data points whilst under the competition treatment (Hurlbert 1984; Underwood 1997) only one plant per pot was statistically analysed, this plant was pre-determined and non-intrusively marked and thus called the ‘target plant’. Thus there were equal numbers ($n = 20$) of the target plants from each population under each competition type treatment and soil Zn treatment.

4.2.2 *Plant Propagation, Harvesting and Sample Preparation:*

Seeds of the CW and GM *R. acetosa* populations were collected from the respective sites in July 2009. All plant propagation took place in the University of Sussex's glasshouses, under temperatures of 15-25 °C, and a 16: 8 hr. (light: dark) photoperiod, using supplementary lighting (Osram SON-T 400 W lamps), and were gently watered from above every day using tap water. For both populations, seeds were sown thinly and evenly in damp vermiculite, under supplementary lighting. Two weeks from the emergence of the first seedling, seedlings with a total of three to four leaves and with uniform root length, were randomly transplanted to plug cells filled with damp John Innes seed compost. After a further two weeks, seedlings from both populations with a total five to six leaves and with uniform root length, were then randomly transferred to a soil Zn treatment and a competition treatment. The Zn-spiked soil treatments consisted of a 15 cm diameter pot with a 6 : 1 silver sand : John Innes No. 2 compost mix, spiked with zinc oxide (ZnO) (Fisher Scientific, Standard Laboratory Grade), homogenously mixed in a cement mixer, to produce a 1500 mg/kg or 45,000 mg/kg concentration based on the DW of the growth medium. Pots were placed on saucers to prevent to the loss of growth medium (and a subsequent decrease in Zn concentration) from watering. To produce the plant pairs within a pot, each seedling was placed 3.75 cm from the side of the pot and hence was 7.5 cm away from its competitor. The target plant was randomly pre-determined and non-intrusively marked before transferal into the soil Zn treatment. Plants were grown under competition in the soil Zn treatments for eight weeks before the shoot material was destructively harvested from the basal node. Root matter was impossible to separate and identify in this experiment. Shoot material was carefully washed under running tap water to ensure that all traces of ZnO were removed from the surface before chemical analysis of foliar Zn concentration. Plant samples were dried at 60°C for 48

hours before having their shoot dry weight (DW) (g) recorded. Herbage samples were ground to a dry, fine powder (<180 µm) using a Fritsch Mini-Pulverisette 23 with stainless steel accessories (Zn-free with negligible contamination from Ni, Cr and Fe), and stored in polythene self-seal bags.

4.2.3 *Foliar Zn Concentration Analysis:*

4.2.3.1 Plant Sample Preparation and Digestion:

Plant samples were prepared and digested as according to the methods presented in chapter 2, section **2.2.6.2.** (*Foliar Zn Concentration Analysis*).

4.2.3.2 Blank Correction, Bias and Precision:

Reagent and matched reagent blank corrections, and the calculation of the bias and precision, were carried out according to the methods and equations presented in chapter 2, section **2.2.6.3.** (*Blank Correction, Bias and Precision*).

4.2.3.3 Flame Atomic Absorption Spectrometry:

F-AAS instrumental parameters, and details of the calibration standards and set-up used, were as described in chapter 2, section **2.2.6.4.** (*Flame Atomic Absorption Spectrometry*).

4.2.4 *Relative Competitive Intensity (RCI):*

The competitive ability was measured using the Relative Competitive Intensity (RCI) as defined and used by Grace (1995) and Weigelt and Jolliffe (2003). The RCI was calculated using block pairs and the following formula as according to Grace (1995):

$$RCI = \frac{(Shoot\ Biomass_{Intra-population} - Shoot\ Biomass_{Inter-population})}{Shoot\ Biomass_{Intra-population}}$$

Negative values denote that when the Target Population 'A' is grown with 'Population A' as a competitor, the target population's shoot biomass is lower, than if Target Population 'A' had been grown with 'Population B'. Hence Population 'A' is a superior competitor for resources compared to Population 'B', and that Population 'A' performs better under inter-population competition than intra-population competition.

Positive values denote that when the Target Population 'A' is grown with 'Population A' as a competitor, the target population's shoot biomass is greater, than if Target Population 'A' had been grown with 'Population B'. Hence Population 'A' is an inferior competitor for resources compared to Population 'B', and that Population 'A' performs better under intra-population competition than inter-population competition.

4.2.5 *Statistical Analysis:*

Assumptions of equal variances and normal distributions for parametric statistics were tested for using Levene's Test and the Kolmogorov-Smirnov test (Zar 2010). Data were transformed if they did not meet these assumptions, and non-parametric statistics were used if transformation did not meet the requirements of the statistical test. All data statistically analysed only came from the target plant in each pot. Pots with either dead target or non-target plants were deemed void and omitted from the analysis.

GLMMs (General Linear Mixed Models) were first used to determine the effect of the random factor 'Block' on the model for a) shoot biomass (DW) and b) LN transformed foliar Zn concentrations. As 'Block' was found to be highly non-

significant it was removed from the model. GLMs (General Linear Models) were then used to determine the effects of soil Zn treatment, population and competition type (fixed factors) and their interaction on a) shoot biomass (DW) and b) LN transformed foliar Zn concentrations. Post-hoc Tukey tests were used to determine which levels were significantly different from each other when $p < 0.05$. A non-parametric version of the GLM, the Scheirer-Ray-Hare test (SRH), was used to determine the effects of soil Zn treatment and population (fixed factors) and their interaction on the ranked values of RCI. Post-hoc Tukey tests on the ranked data were used to determine which levels were significantly different from each other when $p < 0.05$.

4.3 Results:

4.3.1 *Shoot Biomass:*

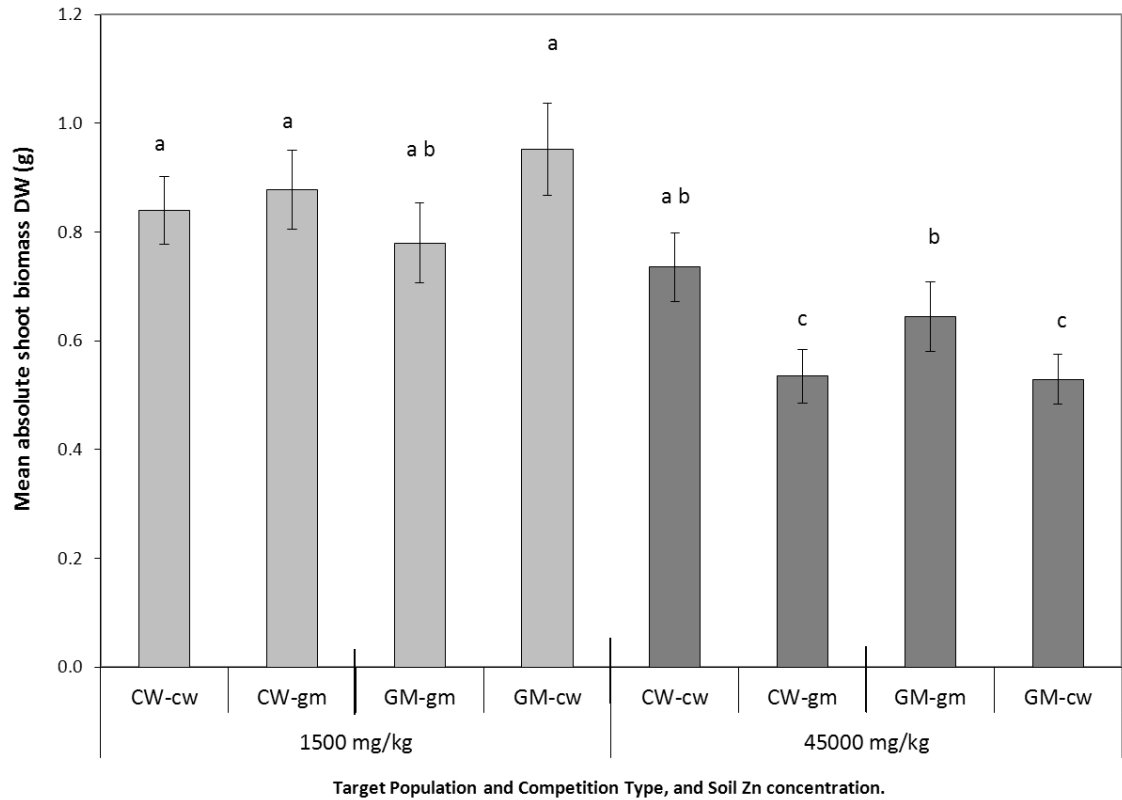


Figure 4- 1: Mean and SE of the shoot biomass (DW) of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. The statistical output of a GLM on the data is displayed in Table 4- 1. A post-hoc Tukey test indicates where significant differences lie.

	GLM for Shoot Biomass (DW).					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Soil Zn Treatment	1	2.49143	2.51349	2.51349	29.83	<0.001
Population	1	0.01874	0.01660	0.01660	0.20	0.658
Competition Type	1	0.03122	0.02807	0.02807	0.33	0.565
Soil Zn Treatment * Population	1	0.02779	0.03054	0.03054	0.36	0.548
Soil Zn Treatment * Competition Type	1	0.68218	0.68669	0.68669	8.15	0.005
Population * Competition Type	1	0.11991	0.12026	0.12026	1.43	0.234
Soil Zn Treatment * Population * Competition Type	1	0.00618	0.00618	0.00618	0.07	0.787
Error	151	12.72509	12.72509	0.08427		
Total	158	16.10253				

Table 4- 1: Statistical output of a GLM on shoot biomass (DW) data, of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. Plants were grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot.

There was a very highly significant main effect of soil Zn treatment and a non-significant main effect of population and competition type on shoot biomass (Figure 4- 1 & Table 4- 1). Hence the shoot biomass of all *R. acetosa* plants was greater when grown at 1500 mg/kg than 45,000 mg/kg and overall the shoot biomasses of both the CW and GM target plants were equal to each other, regardless of the competition type used. Therefore there was no difference in the competitive ability between CW and GM, when grown under inter- or intra-specific pairwise competition.

All interactions were non-significant apart from the soil Zn treatment - competition type interaction which was highly significant. This significant interaction shows that the nature of competition is different at high and low soil Zn concentrations. Target plants grown under inter-population competition in the 1500 mg/kg soil Zn treatment were very highly significantly larger than

those growing under inter-population competition in the 45,000 mg/kg soil Zn treatment. However there was no such trend seen for plants under the intra-population competition treatment.

4.3.2 *Relative Competitive Intensity (RCI):*

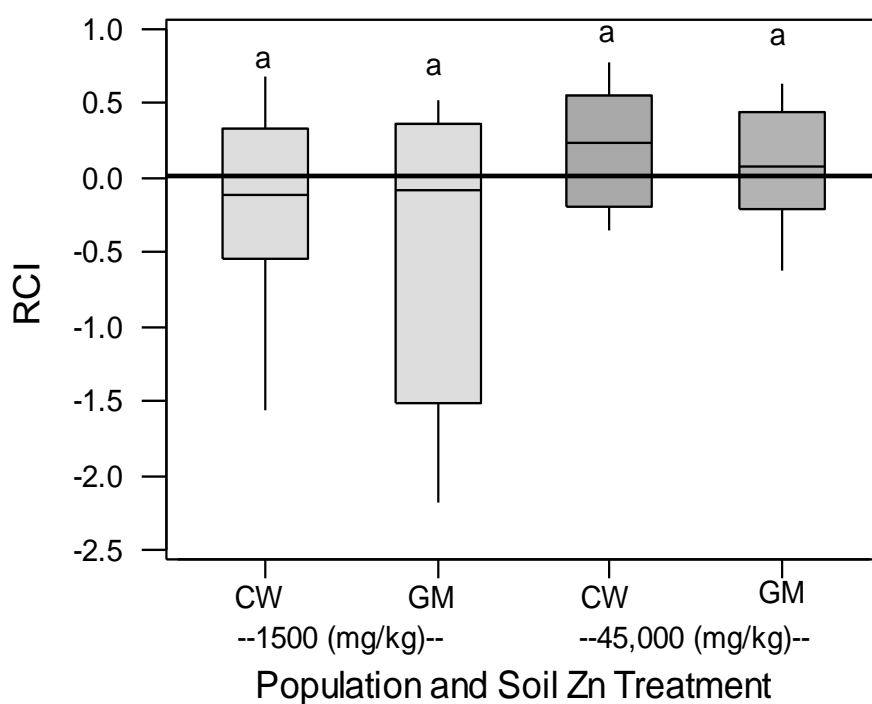


Figure 4- 2: Median and IQR of the Relative Competitive Intensity (RCI) of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. The statistical output of a SRH on the data are displayed in Table 4- 2. A post-hoc Tukey test indicates that no significant differences were found between individual treatments.

SRH Output on Ranked RCI values.						
Source	DF	Adj SS	Adj MS	Adj SS/MS total	Cumulative χ^2 value for df = 1	p value
Soil Zn Treatment	1	3026.9	3026.9	5.7473	0.9835	0.02
Population	1	313.1	313.1	0.5945	0.5593	0.44
Soil Zn Treatment * Population	1	72.6	72.6	0.1378	0.2900	0.71
Total	78	526.667				

Table 4- 2: Statistical output of the SRH test on the ranked Relative Competitive Intensity (RCI) values of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. Plants were grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot.

As seen for the shoot biomass data above, there was a significant main effect of soil Zn treatment on the RCI but not a significant main effect of population or a significant interaction (**Figure 4- 2 and Table 4- 2**). Therefore when grown in the 1500 mg/kg soil Zn treatment, both the CW and GM populations have negative median RCI values, but in the 45,000 mg/kg treatment, both the CW and GM populations have positive median RCI values, although neither was found to be significantly different from zero (data not shown). Thus when grown in the 1500 mg/kg soil Zn treatment under intra-population competition, the shoot biomass of both the CW and GM populations is lower than if they had been grown under inter-population competition. However the opposite is the case for the 45,000 mg/kg treatment - the shoot biomass of both the CW and GM populations grown under intra-population competition is higher than if they had been grown under inter-population competition. Therefore both populations seem to perform slightly better in mixtures when grown in the 1500 mg/kg soil Zn treatment, but when grown in the 45,000 mg/kg soil Zn treatment, both populations perform better in single-population stands.

The non-significant effect of population identity and the non-significant interaction shows that neither the CW nor GM populations were the superior competitor. This agrees with the trends seen for the shoot biomass data – namely that soil Zn concentration (i.e. 1500 mg/kg vs. 45,000 mg/kg) has a greater effect on the competitive ability of a population than the population identity (i.e. CW vs. GM).

4.3.3 *Foliar Zn Concentration:*

4.3.3.4 *Precision and Bias of the Foliar Zn Concentration:*

The overall precision (at 95 % confidence) of the plant samples was within satisfactory levels at 7.9 % (n = 16), and the overall bias of the reference materials was again within satisfactory levels at -3.7 % (n= 4).

4.3.3.5 Foliar Zn Concentration:

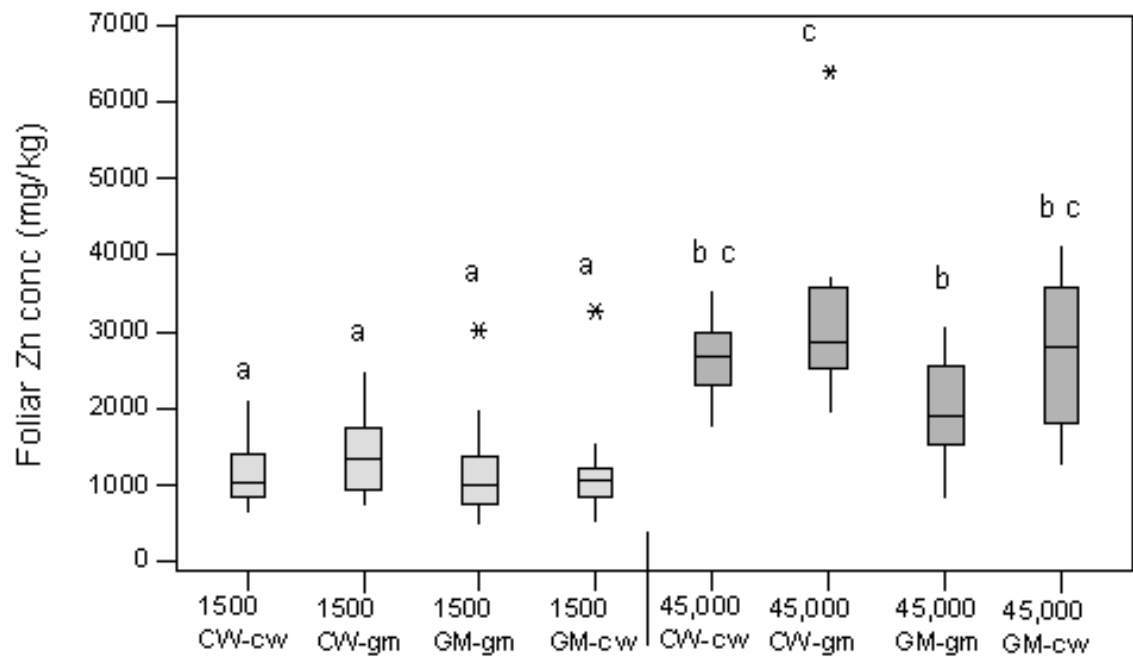


Figure 4- 3: Median and IQR of the foliar Zn concentration (mg/kg) of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. The statistical output of a GLM on the LN-transformed data are displayed in Table 4- 3. A post-hoc Tukey test indicates where significant differences lie. '*' indicate outliers.

	GLM for LN Foliar Zn concentration.					
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Soil Zn Treatment	1	24.1743	24.2236	24.2236	211.98	<0.001
Population	1	1.5035	1.4979	1.4979	13.11	<0.001
Competition Type	1	1.0510	1.0418	1.0418	9.12	0.003
Soil Zn Treatment * Population	1	0.0805	0.0770	0.0770	0.67	0.413
Soil Zn Treatment * Competition Type	1	0.1743	0.1773	0.1773	1.55	0.215
Population * Competition Type	1	0.0000	0.0001	0.0001	0.00	0.981
Soil Zn Treatment * Population * Competition Type	1	0.2845	0.2845	0.2845	2.49	0.117
Error	151	17.2556	17.2556	0.1143		
Total	158	44.5236				

Table 4- 3: Statistical Output of a GLM on LN-transformed foliar Zn concentration data, of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations. Plants were grown at 1500 or 45,000 mg/kg soil Zn concentration. Plants were grown under intra-population competition (2 plants per pot, both from one population), or inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot.

There were very highly significant main effects of soil Zn treatment and population and a highly significant main effect of competition type on foliar Zn concentration (**Figure 4- 3 & Table 4- 3**). None of the 2-way or 3-way interactions were statistically significant. Overall the 45,000 mg/kg soil Zn treatment, CW population and inter-population competition factors produced higher foliar Zn concentrations than their counterparts.

4.3.4 *Summary of Results:*

As seen previously in this thesis, the higher soil Zn treatment had a significant negative effect on shoot biomass compared to the lower soil Zn treatment, and also caused a significant increase in the foliar Zn concentration of both *R. acetosa* populations. Furthermore the highly significantly greater foliar Zn concentration of the CW population compared to the GM population when grown in a standard Zn spiked growth medium was maintained under pairwise competition, regardless of the competition type.

In summary, the soil Zn concentration significantly influenced the outcome of competition, whereas population identity did not significantly. The most productive competition type was affected by the soil Zn concentration. Plants grown under inter-population competition were about 13 % bigger than those under intra-population competition for the 1500 mg/kg soil Zn treatment. However plants grown under inter-population competition were about 23 % smaller than those under intra-population competition for the higher soil Zn treatment. Population differences were not seen for shoot biomass or RCI, but were very highly significant for the foliar Zn concentration, where the CW population was significantly higher than GM (11 % and 18 % respectively for the 1500 and 45,000 mg/kg soil Zn treatments). The competition type imposed also significantly affected the foliar Zn concentration – plants grown under inter-population competition had higher foliar Zn concentrations than those under intra-population competition (11 % and 26 % respectively for the 1500 and 45,000 mg/kg soil Zn treatments).

Therefore the null hypothesis can be rejected as the competitive ability and the foliar Zn concentration of *R. acetosa* was shown to be dependent upon the soil Zn concentration grown at and the type of competition incurred.

4.4 Discussion:

As shown earlier in this chapter, higher soil Zn concentrations have a negative effect on the shoot biomass of both the metallicolous *R. acetosa* populations, and therefore the 45,000 mg/kg Zn treatment can be classed as a greater abiotic stress compared to the 1500 mg/kg Zn treatment. Since plants in both soil Zn treatments had the same initial nutrient levels, watering regime and light levels, stunting in the higher soil Zn treatment is caused by the excessive soil Zn concentration and not resource deficiency, as the higher soil Zn treatment plants were smaller than those in the lower soil Zn treatment. Therefore, even for metallicolous populations, plant growth is decreased under increasing soil Zn concentration and therefore tolerance to the soil toxicity, is at a cost to shoot biomass.

This experiment demonstrated that growth at higher soil Zn concentrations significantly reduced the shoot biomass of metallicolous *R. acetosa* populations. A larger biomass could result in a relatively higher amount of competitive interactions between plants (Keddy 1989), thus the degree of competitive interactions is potentially dependent upon the soil Zn treatment. The significant interaction seen between soil Zn concentration and competition type may be a reflection of this influence of plant size. The shoot biomass in the 1500mg/kg soil Zn treatment was higher in inter-population compared to intra-population competition, yet the higher soil Zn treatment demonstrated the opposite trend. Thus the increased competitive interactions due to plant size at the 1500 mg/kg soil Zn treatment may affect the outcome of competition between the two populations. However as no interaction between population identity and soil Zn concentration or competition type was seen, for the shoot biomass, no population was found to be a superior competitor for resources. Thus, in this experiment, competition for resources had a similar effect on both populations,

and may not have been high enough to produce population differences, or the populations may have subtle physiological differences and thus requirements that allow that coexistence through competition rather than antagonism (Aarssen 1984; Aarssen 1992).

In this experiment the CW population had a higher foliar Zn concentration than GM for both soil Zn treatments under controlled conditions, although this difference is more pronounced for the higher soil Zn treatment. Therefore the CW population is less efficient at restricting Zn uptake into its shoots, especially at higher soil Zn concentrations. Due to the morphological and physiological effects of metal toxicity (Foy, Chaney & White 1978), this may affect the metal tolerance and resource requirement of the populations by potentially requiring more resources to tolerate the elevated soil metal concentrations or by potentially requiring more resources to restrict the Zn uptake into its leaves (Wilson 1988). As no population differences in shoot biomass or the RCI were seen at either soil Zn concentration, the overall resource requirement of these two metal-tolerant populations grown at elevated soil Zn concentrations may be similar. Therefore if the two possible metal tolerance strategies are costly in terms of resources, both populations are able to compensate for it and gain enough resources so that their shoot biomasses do not differ between populations. Conclusive evidence of a trade-off between competitive ability and metal-tolerance is hard to demonstrate however, due to other potential local adaptations to abiotic and biotic conditions (Higgins & Mack 1987; Harper, Smith & Macnair 1997a; Harper, Smith & Macnair 1997b). Potential population differences in resource requirement could be explored further by manipulating the availability of water, light and nutrients, to levels found in the field which may well be lower than those used in this greenhouse experiment, or by extending the experiment so that competition occurred over a longer time period, thus increasing competition for resources as the biomass increases.

In this experiment, the shoot differences between the metallicolous CW & GM populations seen in **Chapter 3**, do not seem to influence their competitive ability, and therefore cannot be used to explain the significant interaction between soil Zn treatment and competition type on competitive ability. Generally higher productivity from inter-specific population competition is thought to be due to differences in the niche requirements of the competing individuals, and if one species or population outperforms the other under competition, then a superior competitive advantage is assumed (Aarssen 1984; Aarssen 1992). Since plants grown under inter-population competition had a higher shoot biomass compared to plants grown in intra-population competition at 1500 mg/kg soil Zn, there is potential difference in the niche resource requirement between the two metallicolous populations. However this potential difference did not produce a superior population under experimental conditions. Conversely for the 45,000 mg/kg soil Zn treatment, a larger shoot biomass was produced under intra-population competition, and therefore greater growth under the 45,000 mg/kg soil Zn treatment could occur under similar niche resource requirements.

Although the pattern of competitive ability interacted with soil Zn treatment, the RCI values for this experiment were very close to zero, and hence the difference seen in competitive ability between soil Zn treatments could be masked by an adequate resource supplies for competing plants under moderate and high soil Zn stress, thus not producing population differences in competitive ability. Measurement of resources such as light intensity and availability, water availability and macronutrient availability in the field and investigation of how the manipulation of these factors in the greenhouse could offer further insight into potential population differences in niche resource requirements. Nevertheless this experiment provides important information on the interaction between biotic and abiotic factors on metal tolerance, regarding

a potential population difference in the niche resource requirement, and the significant interaction between metal toxicity, plant size and competitive interactions. The potential for the interaction between biotic and abiotic stress factors on Zn contaminated soils is now discussed.

As mentioned above, growth under lower resource availability or over a longer time-period, could demonstrate a greater competitive interaction as resource availability decreased. This is supported by the difference in shoot biomass and the positive and negative RCI found between the soil Zn treatments. As shown by Barrutia *et al.* (2009), increased access to nutrients increases the shoot biomass of both metallicolous and non-metallicolous populations of *R. acetosa* grown under metal stress comparable to bare spoil heaps, therefore nutrient availability is a potential limiting factor to shoot biomass and metal tolerance. Hence, the resource requirement and uptake of metallicolous populations of *R. acetosa* on a sparsely vegetated mining site would probably not be selected for by biotic factors such as resource competition, as competitive interactions with other plants would be relatively low, but possibly instead by abiotic factors such as soil metal toxicity and nutrient status. However, under lower Zn contamination, the presence of a higher abundance and diversity of individuals could cause the resource requirement and uptake of metallicolous populations of *R. acetosa* to be instead selected for by biotic factors, such as increased competition for light and nutrients, due to the potential increased influence of neighbours on resource availability (Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975; Higgins & Mack 1987). This may have not been clearly demonstrated in this experiment as two metallicolous populations were used, adapted to growth on highly contaminated soils with a low nutrient status, which whilst differing in their ability to restrict Zn uptake into their shoots, did not seem to differ in their resource uptake and requirements, possibly due to their current adaptation to low resource conditions and short time period of the

experiment. Therefore differences in the competitive ability for resources of metalicolous populations are probably affected by life-history traits and adaptation that have evolved to the high toxicity and nutrient status of mining sites. It has been suggested that differences in the life history traits of metalicolous and non-metallicolous ecotypes of *Thlaspi caerulescens* have been selected for by difference in the Zn uptake and regulation cellular process due to the site soil Zn concentration (Assunção, Schat & Aarts 2003; Jiménez-Ambriz *et al.* 2007). Differences in several life history traits in metalicolous populations of *T. caerulescens* have also been found, due to environmental differences in the exposed and sheltered areas habitats they were sampled from (Dechamps *et al.* 2011).

Although the superior ability of the GM population to restrict Zn shoot uptake was demonstrated, the potential cost of this trait through a higher resource requirement, and hence population differences in shoot biomass competitive ability were not shown here. Growth under higher soil Zn concentrations may result in an increased resource requirement for increased Zn tolerance in metalicolous populations of *R. acetosa*, whereas the increased relative growth under moderate contamination may result in an increased resource requirement for growth and maintenance. Qualitative and quantitative changes in secondary compounds due to metal stress, may also have an impact on the resource requirement of *R. acetosa*, as seen with Al (Tolrà *et al.* 2005) and Zn (Mathys 1977), and Zn, Pb and Cd (Epelde *et al.* 2010). However the differences and interaction between the specific resource requirements of each metalicolous population and their relationship to local site adaptation are unknown.

4.5 Conclusion:

Previous experiments conducted under controlled conditions using a standard growth medium displayed shoot differences between metalicolous *R. acetosa* populations. This experiment investigated the relationship between Zn uptake and competitive ability through these population differences, and hence their overall metal tolerance ability. Growth under high soil Zn concentrations reduced the shoot biomass of both metalicolous populations, indicating a cost to survival in highly contaminated soils, even for metalicolous populations. However when grown under competition, population differences were not seen for shoot biomass but they were seen for foliar Zn concentration. Hence the potential population differences in resource requirement for metal tolerance did not produce population differences in competitive ability. In conclusion, pair-wise competitive growth under moderate soil Zn contamination produced conditions that allow the population differences in the competitive ability and Zn uptake between the CW and GM populations to complement each other under moderate Zn contamination by producing greater growth when grown in inter-population competition. Hence neither population was the superior competitor. Reduced growth under higher soil Zn concentrations seemed to reduce the competitive interactions so far as that intra-population competition increased shoot growth and again neither population was the superior competitor. Therefore in this experiment population differences seem to allow co-existence between competing individuals. Additionally, it is suggested that further work also investigates populations which contrast in their shoot and root biomass as well as their foliar Zn concentration and CF, to determine how this interacts with competitive ability. This could involve the use of the BR population, which had a lower shoot biomass compared to the GM population shown in **Chapter 3 Fig. 3-4** and thus might be a potentially weaker competitor for resources to support this lower biomass. Manipulation of the availability of light, water and macronutrient concentration to levels seen at non-metalliferous

sites and the metalliferous sites used in this thesis, could additionally provide insight on the selective pressures acting at each sites and how they interact with metal tolerance and competitive ability. Nevertheless, when comparing the CW and GM populations, a longer time period of competition and lower resource availability may additionally highlight potential population differences and their interaction with competitive ability and metal tolerance.

Chapter 5: Effects of Helix aspersa Herbivory on the Competitive Ability, Foliar Zinc Concentration and Palatability of two Metallicolous Populations of Rumex acetosa, grown at Contrasting Soil Zinc Concentrations.

5.1 Introduction:

Herbivory may decrease productivity and fitness, cause mortality, or promote compensatory regrowth, all of which produce major physiological changes within the plant (Crawley 1997). The influence of biotic interactions on metallicolous plant populations is not solely limited to competition, but is also affected by and interacts with herbivory in the field, potentially altering the competitive dominance (Louda, Keeler & Holt 1990; Crawley 1997; Hawkes & Sullivan 2001). For instance Bentley & Whittaker (1979) found that beetle grazing altered the competitive interactions between two *Rumex* spp. The beetle *Gastrophysa viridula* prefers *R. obtusifolius* in choice trials, and when grown under inter-specific competition without grazing, *R. obtusifolius* is competitively superior to *R. crispus*. However when grazing and competition are combined, the competitive ability of *R. obtusifolius* is reduced sufficiently to prevent mortality of *R. crispus*.

Thus herbivory may also additionally interact with the competitive ability of a plant to acquire limited resources, especially when the environment constrains the response of the plant (Louda, Keeler & Holt 1990; Hawkes & Sullivan 2001). The mechanisms behind metal tolerance and foliar metal accumulation may be regarded as costly by reducing the fitness of the plant, through energy expenditure and the adaptations required, potentially producing a trade-off between metal-tolerance and fitness, although this is hard to demonstrate conclusively (Baker 1987; Wilson 1988; Ernst, Schat & Verkleij 1990; Harper,

Smith & Macnair 1997a; Harper, Smith & Macnair 1997b; Ernst 2006; Verbruggen, Hermans & Schat 2008; Maestri *et al.* 2010). Therefore changes in the plant chemistry from elevated soil metal concentrations (Tolrà *et al.* 2001; Tolrà *et al.* 2005) may also affect herbivore preference and therefore the competitive ability. Changes in the plant chemistry from herbivory may also affect the competitive ability for resources. Davis & Boyd (2000) found that herbivory did not increase Ni concentration in a Ni hyperaccumulator, but herbivory did increase glucosinolate concentration and nitrogen concentration, thus reducing the carbon: nitrogen ratio of the plant and potentially affecting its resource requirements. Additionally, there is a potential competitive advantage to metal uptake as higher shoot metal concentrations may protect the plant from herbivory due to deterrence and reduced herbivore performance (Boyd & Martens 1992). Thus, the potential costs of metal uptake in terms of reduced growth from metal-toxicity may be outweighed by the benefits to growth gained through protection against herbivory; thus the competitive ability of plants in metallicolous communities will reflect their ability to uptake the resources required for both survival and defence.

Chapter 3 demonstrated that population preference by *Helix aspersa* was dependent upon both the population identity and the soil zinc (Zn) concentration at which it was growing. Thus populations of *R. acetosa* may respond differently to increasing soil Zn concentration, which affects their leaf palatability. **Chapters 3 and 4** also demonstrated that growth at higher soil Zn treatments reduced the shoot biomass of metallicolous populations, and therefore survival under higher soil concentrations is at a cost to productivity. Although the potential differences between the metallicolous populations of *R. acetosa* affected the competitive interactions between soil Zn treatments in **Chapter 4**, neither population was the superior competitor and population differences seem to allow co-existence under competing individuals.

Competition over a longer time period may highlight population differences and their interaction with competitive ability and metal tolerance, to ascertain if one of the populations is competitively superior to the other. The interaction between herbivory and competitive ability for resources, when under Zn stress, is explored further in this chapter.

5.1.1 *Chapter Aim:*

The aims of this chapter were two-fold, and are linked with the results of the previous chapter. Using a longer time period of four months, the influence of soil Zn concentration on the competitive ability of the two metalicolous populations of *R. acetosa*, when grown under inter-specific competition, was investigated under the absence and presence of *H. aspersa* herbivory. A superior competitor for resources (light, water and nutrients, defined by a greater shoot biomass) may be positively or negatively affected by herbivory. Plants which may have increased resource capture ability for improved metal tolerance and productivity may also have an increased palatability due to increased plant quality or a reduced palatability due to increased levels of defence compounds. Thus the presence of herbivory may alter the outcome of their competitive dominance.

5.1.1.1 Null Hypotheses:

1. In the absence of herbivory, there is no statistically significant difference in the competitive ability (defined by the shoot biomass), overall percentage herbivore damage and foliar total Zn concentration of the CW and GM populations when grown under inter-population competition, at 1500 mg/kg or 45,000 mg/kg soil Zn concentration.
2. In the presence of herbivory, there is no statistically significant difference in the competitive ability (defined by the shoot biomass), overall

percentage herbivore damage and foliar total Zn concentration of the CW and GM populations when grown under inter-population competition, at 1500 mg/kg or 45,000 mg/kg soil Zn concentration.

5.2 Methods:

5.2.1 *Experimental Design:*

This experimental design consists of three treatments imposed on the pot, each with two levels (shown in brackets): soil Zn treatment (1500 mg/kg and 45,000 mg/kg), population identity (CW and GM) and herbivory treatment (presence (+H) and absence (-H)). Pots were randomly arranged in blocks, consisting of one pot per treatment combination. 20 blocks were used and were designated as a random factor. Therefore a fully randomised, 3 x 2 fully factorial block design was used. This experiment looks at the competitive interaction between the CW and GM populations using a pairwise competition design based on the de Wit replacement series (Firbank & Watkinson 1990), each pot consisting of two plants per pot – one from the CW population and one from GM. The position of each population was randomly assigned to the left or right side of the pot in advance to avoid potential positional bias. To ensure statistical independence of data points whilst under the competition treatment (Hurlbert 1984; Underwood 1997) only one plant per pot was statistically analysed, this plant was pre-determined and non-intrusively marked and thus called the 'target plant'. Thus there were equal numbers ($n = 20$) of the target plant from each population for each treatment.

5.2.2 *Plant Propagation:*

Seeds of the CW and GM *R. acetosa* populations were collected from the respective sites in July 2009. All plant propagation took place in the University

of Sussex's glasshouses, under temperatures of 15-25 °C, and a 16: 8 hr. (light: dark) photoperiod, using supplementary lighting (Osram SON-T 400 W lamps), and were gently watered from above every day using tap water. For both populations, seeds were sown thinly and evenly in damp vermiculite, under supplementary lighting. Two weeks from the emergence of the first seedling, seedlings with a total of three to four leaves and with uniform root length, were randomly transplanted to plug cells filled with damp John Innes seed compost. After a further two weeks, seedlings from both populations with a total five to six leaves and with uniform root length, were then randomly transferred to a soil Zn treatment and a competition treatment. The Zn-spiked treatments consisted of a 15 cm diameter pot with a 6 : 1 silver sand : John Innes No. 2 compost mix, spiked with zinc oxide (ZnO) (Fisher Scientific, Standard Laboratory Grade), homogenously mixed in a cement mixer, to produce a 1500 mg/kg or 45,000 mg/kg concentration based on the DW on the growth medium. Pots were placed on saucers to prevent to the loss of growth medium (and a subsequent decrease in Zn concentration) from watering. To produce the plant pairs within a pot, each seedling was placed 3.75 cm from the side of the pot and hence was 7.5 cm away from its competitor. The target plant was randomly pre-determined and non-intrusively marked before transferal into the soil Zn treatment. Plants were grown under competition in the soil Zn treatments for seven weeks before the herbivory treatment was imposed to ensure that the plants had established enough biomass for the herbivores to consume.

5.2.3 *Herbivores:*

Adult *Helix aspersa* snails were again collected from residential areas and allotments of Southern England, where molluscicides had not been knowingly applied in the previous year. Therefore snails did not have experience of metalliferous sites or metalicolous plants. Adults were identified by the

presence of a 'lip' on the shell (Kerney & Cameron 1979). Snails were maintained in glass tanks lined with compost, misted daily, and kept under natural lighting at 18 °C. Snails had been previously observed to eat non-metallicolous *Rumex* spp. Snails were denied access to food for four days before being placed in the pots, to ensure that their appetites were not sated to allow feeding on the plants.

5.2.4 *Herbivory Treatment:*

Plants were grown in the standard inter-population competition design as described above until week 7 of growth, where half were allocated to the 'herbivory present' treatment (+H) and half to the 'herbivory absent' treatment (-H). Four *Helix aspersa* adult snails (shell height mean (SE) = 29.55 mm (+/- 0.09 mm), shell breadth mean (SE) = 22.26 mm (+/- 0.07 mm), was added to the centre of all pots in the (+H) treatment. All plants (+H and -H treatments) were caged with two 30 cm stakes pushed into the pot, and a voile sleeve secured over the stakes and completely around the pot. This allowed the snails' free access to both plants. The plants were then watered from below on an *ad lib* basis. Cages were misted from above to provide sufficient moisture for the snails and the greenhouse was kept at 18 °C with no supplementary lighting. After adding the snails, each pot was monitored for 3 days to ensure that at least 5% of the plant had been damaged. If not it was replaced by another set of starved adult snails. As the plant consumption was not excessive, possibly due to the high foliar Zn concentration of the plants, the both +H and -H plants were left to grow for a further four months, to allow enough time for the presence of herbivory to have an effect on shoot biomass and for the plants to compete for resources.

5.2.5 *Estimation of Percentage Damage to Plant:*

Before harvesting, the percentage damage to each leaf was recorded and the total number of leaves per plant counted so that the percentage damage to the plant could be calculated and used in the statistical analysis. The final leaf count included leaf stalks (where leaf blades had been completely consumed). The percentage damage to the plants was calculated as:

$$\text{Percentage damage to plant} = \frac{\Sigma \% \text{ damage to each leaf}}{\text{final leaf count}}$$

5.2.6 *Plant Harvesting and Sample Preparation:*

After a total of six months (two months establishment plus four months under the herbivory treatment), the shoot material was destructively harvested from the basal node. Root matter was impossible to separate and identify. Shoot material was carefully washed under running tap water to ensure that all traces of ZnO were removed from the surface before chemical analysis of foliar Zn concentration. Herbage samples were dried at 60°C for 48 hours before having their dried weight (DW) (g) recorded. Herbage samples were ground to a dry, fine powder (<180 µm) using a Fritsch Mini-Pulverisette 23 with stainless steel accessories (Zn-free with negligible contamination from Ni, Cr and Fe), and stored in polythene self-seal bags.

5.2.7 *Foliar Zn Concentration Analysis:*

5.2.7.2 *Sample Preparation and Digestion:*

Plant samples were prepared and digested as according to the methods presented in chapter 2, section 2.2.6.2. (*Foliar Zn Concentration Analysis*).

5.2.7.3 Blank Correction, Precision and Bias:

Reagent and matched reagent blank corrections, and the calculation of the bias and precision, were carried out according to the methods and equations presented in chapter 2, section 2.2.6.3. (Blank Correction, Bias and Precision).

5.2.7.4 Flame Atomic Absorption Spectrometry:

F-AAS instrumental parameters, and details of the calibration standards and set-up used, were as described in chapter 2, section 2.2.6.4. (Flame Atomic Absorption Spectrometry).

5.2.8 *Statistical Analysis:*

Assumptions of equal variances and normal distributions for parametric statistics were tested for using Levene's Test and the Kolmogorov- Smirnov test (Zar 2010). Data was transformed if it did not meet these assumptions, and non-parametric statistics were used if transformation did not meet the requirements of the statistical test. Only data from the target plant in each pot was statistically analysed. Pots with either dead target or non-targets plants were deemed void and omitted from the analysis.

GLMMs were first used to determine the effect of block (random factor) on the model for a) LN transformed shoot-biomass, b) LN transformed foliar Zn concentrations, and c) overall % damage to the +H plants. As block was found to be highly non-significant it was removed from the model. GLMs were used to determine the effects of soil Zn treatment, herbivory treatment and inter-population pairing (subsequently denoted as 'pairing' in the statistical outputs) (fixed factors) and their interaction on a) LN transformed shoot-biomass and b) LN transformed foliar Zn concentrations. Post-hoc Tukey tests were used to

determine which levels were significantly difference from each other when $p < 0.05$. A non-parametric version of the GLM, the Scheirer-Ray-Hare (SRH) test, was used to determine the effects of soil Zn treatment and inter-population pairing (fixed factors) and their interaction on the ranked values of percentage damage. Post-hoc Tukey tests on the ranked data were used to determine which levels were significantly difference from each other when $p < 0.05$. Spearman's rank correlations were used to determine the strength of the relationship between the foliar Zn concentration and percentage damage for each combination of treatment levels.

5.3 Results:

5.3.1 *Shoot Biomass:*

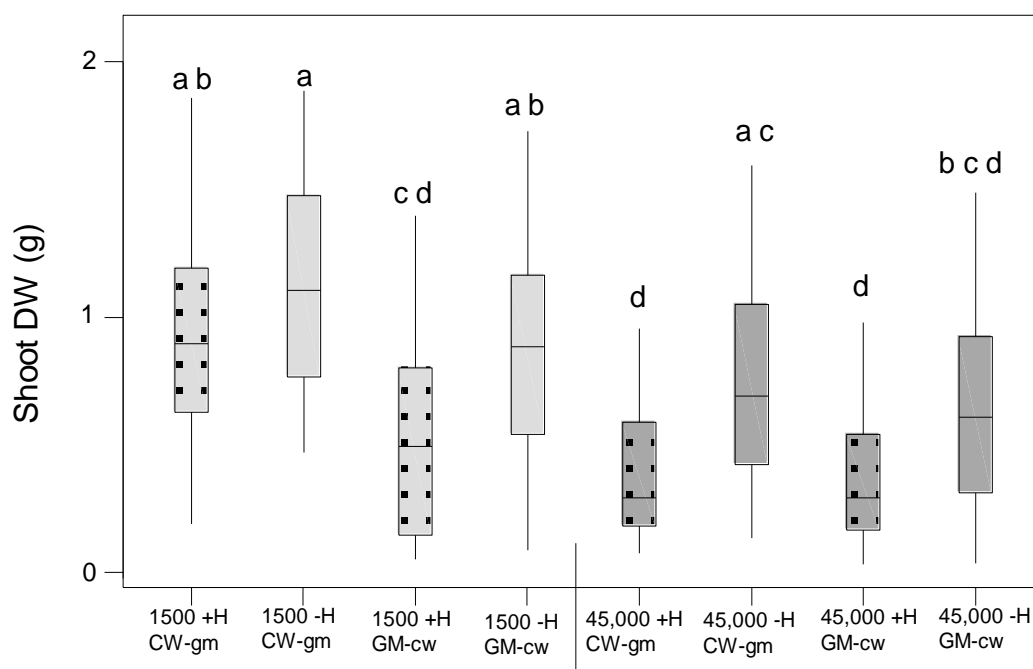


Figure 5- 1: Median and IQR of the shoot biomass dry weight (g) of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown in the presence (+H, dotted boxes) or absence (-H, plain boxes) of *Helix aspersa* herbivory and at either 1500 or 45,000 mg/kg soil Zn concentration. The statistical output of a GLM on LN transformed data is displayed in Table 5- 1. A post-hoc Tukey test indicates where significant differences lie.

	GLM for LN Shoot Biomass (DW).					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Soil Zn treatment	1	11.9979	11.8227	11.8227	21.31	< 0.001
+/- H	1	14.0742	14.1507	14.1507	25.50	< 0.001
Pairing	1	6.5293	6.5368	6.5368	11.78	0.001
Soil Zn treatment * +/- H	1	0.2973	0.2919	0.2919	0.53	0.469
Soil Zn treatment * Pairing	1	2.0931	2.1038	2.1038	3.79	0.053
+/- H * Pairing	1	0.1630	0.1679	0.1679	0.30	0.583
Soil Zn treatment * +/- H * Pairing	1	1.0953	1.0953	1.0953	1.97	0.162
Error	165	91.5535	91.5535	0.5549		
Total	172	127.8053				

Table 5- 1: Statistical Output of a GLM on LN transformed shoot biomass (DW) data, of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot (Pairing Treatment). Plants were grown in the presence (+H) or absence (-H) of *Helix aspersa* herbivory and at either 1500 or 45,000 mg/kg soil Zn concentration.

The highly significant main effects of soil Zn treatment, herbivory treatment and pairing (**Figure 5- 1 & Table 5- 1**), show that overall *R. acetosa* shoot biomass is significantly lower when grown in the 45,000 mg/kg soil Zn treatment compared to the 1500 mg/kg soil Zn treatment, that the presence of herbivory over four months (+H) had a significant negative effect on the shoot biomass of the plants, compared to those grown in the absence of herbivory (-H), and that overall, the CW population had a significantly greater biomass than the GM population when grown under inter-population pairwise competition for six months.

This suggests that generally the CW population is superior competitor for resources compared to the GM population. However there was a significant interaction between the pairing and soil Zn treatments. Shoot biomass was not significantly different between the CW and GM population when grown at the 45,000 mg/kg soil Zn treatment, but was so at the 1500 mg/kg treatment, with

CW target plants having a highly significant greater biomass than GM target plants. Therefore the competitive ability of CW against GM was greater at the 1500 than at the 45,000 mg/kg soil Zn treatments and thus population differences in the competitive ability of *R. acetosa* are reduced under higher soil Zn concentrations.

A non-significant interaction was seen between the pairing & herbivory treatments, and between the soil Zn & herbivory treatments. Thus the presence of herbivory caused the same magnitude of reduction in biomass for both CW and GM grown under inter-population pairwise competition and hence the competitive superiority of CW was not dependent upon the presence or absence of herbivory.

5.3.2 Percentage Damage from Herbivory:

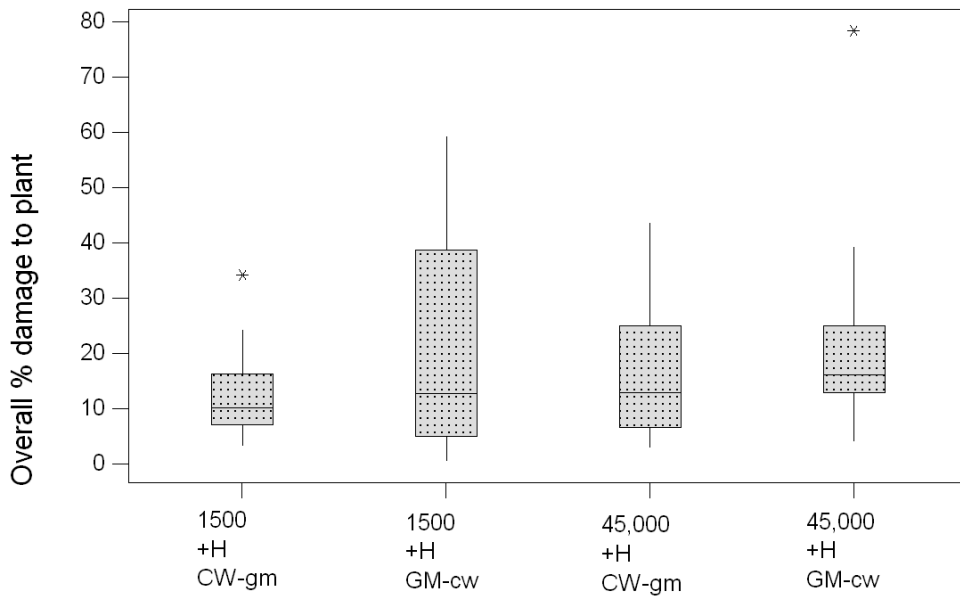


Figure 5- 2: Median and IQR of the overall percentage damage by *Helix aspersa* of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Plants were grown in either 1500 or 45,000 mg/kg soil Zn concentration. Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. A SRH test found no significant differences or interaction between pairing and soil Zn treatment, the results of which are displayed in Table 5- 2. '*' indicate outliers.

SRH Output on Ranked Percentage Damage to Plant						
Source	DF	Seq SS	Adj MS	SS/MS total	Cumulative χ^2 value for df = 1	p value
Soil Zn treatment	1	1228.2		1.97	0.8396	0.1604
Pairing	1	1388.3		2.23	0.8644	0.1356
Soil Zn treatment * Pairing	1	5.6		0.01	0.0756	0.9244
Total	85	52993.5	623.453			

Table 5- 2: Statistical output of the SRH test on the ranked overall percentage damage by *Helix aspersa* values of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot (Pairing Treatment). Plants were grown in either 1500 or 45,000 mg/kg soil Zn concentration.

The median overall percentage damage to the target plants after 4 months of snail presence was relatively low and not statistically different between any of the treatments (**Figure 5- 2 & Table 5- 2**).

5.3.3 Foliar Zn Concentration:

5.3.3.1 Precision and Bias:

The overall precision (at 95 % confidence) of the plant samples was within satisfactory levels at 4.5 % (n = 22), and the overall bias of the reference materials was again within satisfactory levels at 10.7 % (n= 4). A few samples could not be analysed for their total foliar Zn concentration due to their insufficient biomass.

5.3.3.2 Foliar Zn Concentration:

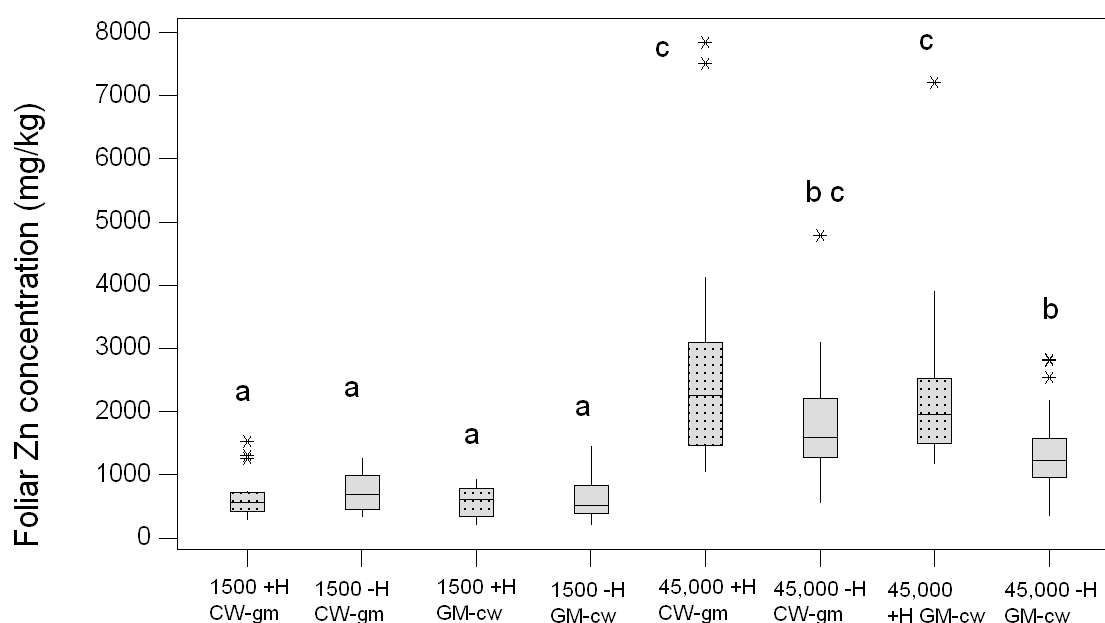


Figure 5- 3: Median and IQR foliar Zn concentration (mg/kg) (DW) of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown in the presence (+H, dotted boxes) or absence (-H, plain boxes) of *Helix aspersa* herbivory and at either 1500 or 45,000 mg/kg soil Zn concentration. The statistical output of a GLM on LN transformed data is displayed in Table 5- 3. A post-hoc Tukey test indicates where significant differences lie. '*' indicate outliers.

	GLM for LN Foliar Zn concentration					
Source	DF	Seq SS	Adj SS	Adj MS	F	P value
Soil Zn treatment	1	50.8698	51.0686	51.0686	219.28	<0.001
+/- H	1	1.2512	1.2741	1.2741	5.47	0.021
Pairing	1	1.0236	1.2327	1.2327	5.29	0.023
Soil Zn treatment * +/- H	1	3.0463	3.0207	3.0207	12.97	<0.001
Soil Zn treatment * Pairing	1	0.0060	0.0059	0.0059	0.03	0.873
+/- H * Pairing	1	0.2192	0.2192	0.2192	0.94	0.333
Soil Zn treatment * +/- H * Pairing	1	0.0022	0.0022	0.0022	0.01	0.923
Error	162	37.7286	37.7286	0.2329		
Total	169	94.1468				

Table 5- 3: Statistical Output of a GLM on LN transformed foliar Zn concentration data, of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot (Pairing Treatment). Plants were grown in the presence (+H) or absence (-H) of *Helix aspersa* herbivory and at either 1500 or 45,000 mg/kg soil Zn concentration.

The significant main effects of soil Zn treatment, herbivory treatment and pairing show that overall *R. acetosa* foliar Zn concentration is highly significantly greater when grown in the 45,000 mg/kg soil Zn treatment compared to the 1500 mg/kg soil Zn treatment, is significantly greater in the presence of herbivory than in the absence of it, and that the foliar Zn concentration of CW is significantly greater than GM (**Figure 5- 3 & Table 5- 3**).

When grown in the 1500 mg/kg soil Zn treatment, neither the herbivory treatment or the pairing treatment had a significant effect on the foliar Zn concentration on both the CW and GM populations of *R. acetosa*. However there was a highly significance difference in foliar Zn concentration between the herbivory treatments within the 45,000 mg/kg soil Zn treatment, as shown by the highly significant soil Zn treatment - herbivory interaction. Under the more

stressful, high soil Zn concentration, the foliar Zn concentration of both populations increased under the presence of herbivory. This increase in foliar Zn concentration from the presence of herbivory compared to the absence of it was significant for the GM population only.

5.3.3.3 Relationship between Foliar Zn Concentration and Overall Percentage

Damage:

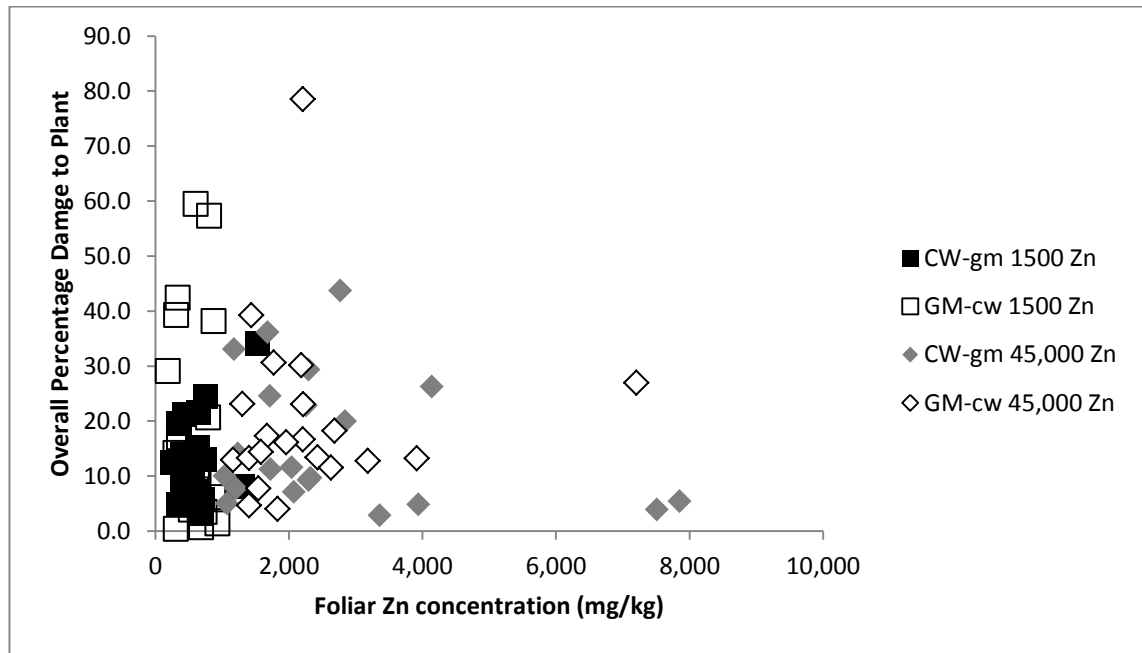


Figure 5- 4: Relationship between the foliar Zn concentration (mg/kg) (DW) vs. overall percentage damage of the Clough Wood (CW) and Gang Mine (GM) *Rumex acetosa* populations, grown under inter-population competition (2 plants per pot, 1 from each population). Data are from the pre-defined target plant within the pot. Capital letters denote the target plant within the pairing. Plants were grown at either 1500 or 45,000 mg/kg soil Zn concentration and were in the presence of *Helix aspersa* herbivory for 4 months. Spearman's Rank: a) CW-gm 1500 Zn $r_s = 0.137$, $P = 0.542$; b) GM-cw 1500 Zn $r_s = -0.213$, $P = 0.368$; c) CW-gm 45,000 Zn $r_s = 0.280$, $P = 0.219$; d) GM-cw 45,000 Zn $r_s = 0.154$, $P = 0.506$.

There was no statistically significant relationship between the foliar Zn concentration and percentage damage from herbivory (**Figure 5- 4**). Although the foliar Zn concentrations were significantly lower for the 1500 mg/kg soil Zn treatment and GM population (**Table 5- 3**) the levels of herbivory did not differ between the soil Zn treatments nor population used and thus the consequent foliar Zn concentrations produced.

5.3.4 *Summary of Results:*

When grown under pairwise inter-population competition for six months, growth under the 45,000mg/kg soil Zn treatment caused a highly significant reduction (by about 38 %) in the shoot biomass, and a highly significant increase in the foliar Zn concentration (by about 215 %) of metallicolous *R. acetosa* populations, compared to the 1500 mg/kg treatment. The presence of herbivory also caused a highly significant reduction (by about 30 %) in the shoot biomass and a significant increase the foliar Zn concentration (by about 37 %), compared to the absence of herbivory. The foliar Zn concentration significantly interacted with the soil Zn treatment and herbivory treatment – *R. acetosa* grown under the 45,000 mg/kg soil Zn treatment and the presence of herbivory had a significantly higher foliar Zn concentration (by about 56 %) compared to those under the absence of herbivory, but this difference was not significant for the 1500 mg/kg soil Zn treatment. The percentage damage to the plants by *H. aspersa* generally ranged between 5 to 40 % and was not significantly affected by population identity or soil Zn treatment.

Furthermore, pairwise inter-population competition for six months in a standard Zn spiked growth medium, caused the CW population to have a highly significantly shoot biomass and a significantly greater foliar Zn concentration than GM (by about 26 and 16 % respectively). This population difference in competitive superiority significantly interacted with the soil Zn concentration – the shoot biomass of CW compared to GM was not significantly greater for the 45,000 mg/kg soil Zn treatment, but was highly significantly greater for the 1500 mg/kg soil Zn treatment (by about 32 %). No interaction between population and soil Zn treatment for the foliar Zn concentration was seen however.

5.4 Discussion:

The aims of this chapter were two-fold, and link with the results of **Chapter 4** which investigated the effect of competition type on the competitive ability and foliar Zn concentration of two metalicolous *R. acetosa* populations. The previous chapter found that although competition type interacted with soil Zn concentration to affect shoot biomass, population differences in competitive ability were not significant. However, as the plants were only under competition for eight weeks, the amount of competition experienced by the two plants may have been limited and thus the population differences seen earlier in this thesis may have not affected the competitive outcome. By growing the two populations under inter-population competition for a longer period of time (a total of six months), under the absence and presence of herbivory, this chapter investigated if the competitive outcome was the same under the absence of herbivory, and if the presence of herbivory altered this or not.

The population identity had a significant effect on both the competitive ability and foliar Zn concentration under the absence of herbivory, thus rejecting null hypothesis 1. This demonstrated that a longer period of competition was required for population differences to affect the competitive outcome when under pairwise-competition.

Generally the CW population was the superior competitor for resources compared to the GM population when grown under inter-population pairing. This did not interact with the presence or absence of herbivory but did interact with soil Zn concentration – a significant difference in competitive ability was seen between the two populations at 1500 mg/kg but not at the 45,000 mg/kg treatment. Hence population variation in competitive ability is more distinct under moderately contaminated soil Zn concentrations than under highly

contaminated soils, but is not affected by the presence or absence of *H. aspersa* herbivory.

5.4.1 *Competition under the Absence of Herbivory:*

The distinction in the competitive ability seen at the moderate, but not at the high soil Zn concentration, is probably due to the lower abiotic stress and consequential size difference and absolute resource requirements (Wilson 1988; Barrutia *et al.* 2009; Maestri *et al.* 2010). As the plants grown in the 1500 mg/kg soil Zn treatment were twice the size of those grown in the 45,000 mg/kg soil Zn treatment, we can conclude that the less stressful abiotic conditions of the former caused a higher growth rate and therefore had a greater resource requirement and potentially experienced more competitive interactions than those in the higher soil Zn treatment.

The restricted growth under higher soil Zn concentrations may be due to the greater physiological adjustments needed to tolerate higher Zn concentrations (Baker 1978; Baker 1987; Ernst 2006). These include the restricted movement of Zn from the soil into the plant, and production of phytochelatins, metallothioneins and organic acids such as malate and citrate in the leaves (Schat, Llugany & Bernhard 2000). These functions may be costly and so impact shoot biomass, although the detailed cost of these mechanisms is unknown (Punz & Sieghardt 1993). Therefore, when growing under highly Zn contaminated soils, the metal stress is significant enough to reduce *R. acetosa* biomass production significantly, thus reducing the competitive interactions between the plants and hence mask any differences in competitive ability between individuals. Lower soil Zn concentrations however, promote differentiation in the competitive ability of *R. acetosa* due to an increased biomass and therefore increased competition for resources.

The difference in the competitive ability of the two populations may also be influenced by metal tolerance processes as well from the amount of competition present. Population differences were also seen for the foliar Zn concentration as well as the competitive ability. In this chapter and throughout this thesis, the CW population has demonstrated a significantly higher foliar Zn concentration than the GM population, when grown in the same standard Zn-spiked growth medium mix and volume under controlled conditions. This was also seen in the field, where the concentration factor (i.e. the ratio between the foliar Zn and soil Zn concentrations) of the CW *R. acetosa* population was significantly greater than the GM population. Hence the two *R. acetosa* populations potentially differ in their ability to restrict the uptake of Zn into their aerial parts – the CW population with its higher foliar Zn concentration and higher concentration factor compared to the GM population is potentially unable to restrict the movement of Zn from the soil to its shoots as much as GM is able to. Therefore, GM's potentially greater restriction ability could be more costly to the plant and therefore result in a smaller biomass. This is theory supported by this study as the GM population has a lower shoot biomass and lower foliar Zn concentration compared to CW for both soil Zn treatments, when grown under inter-population competition for a six-month period.

Meerts *et al.* (2003) investigated the competitive ability of metallicolous and non-metallicolous ecotypes of the hyperaccumulator *Thlaspi caerulescens*, grown on varying solubilities of Zn spiked growth medium. Where Zn availability was manipulated to be very accessible using ZnSO₄, the non-metallicolous ecotype had a very highly significantly lower shoot biomass, and was competitively weaker when grown in competition with the metallicolous ecotype. When grown with very low Zn availability, the non-metallicolous population had the greater shoot biomass and competitive ability. In growth medium with limited Zn solubility, no significant differences were found. They concluded that the

lower Zn tolerance of the non-metallicolous ecotype results in a decreased competitive ability, when there is a high bioavailable Zn concentration. The above study provides support for how metal tolerance and competitive ability are inversely related. However *T. caerulescens* is thought to have an inherently high Zn requirement due to a variety of physiological mechanisms (Shen, Zhao & McGrath 1997), and evolution of hyperaccumulation in *Thlaspi* spp. may have resulted from selection for increased Zn uptake capacity on Zn-poor soils (Meerts *et al.* 2003). However as a pseudometallophyte, *R. acetosa* is usually found on fertile grassland (Salt & Whittaker 1998) and on a range of soil types (Lousley & Kent 1981). Therefore *R. acetosa* may have been able to colonise mining sites from zonal areas by restricting the movement of Zn into its shoots to enable Zn tolerance instead. This is seen in most metal-tolerant populations (Baker & Walker 1990), such as the pseudometallophytes *Agrostis tenuis* (Dahmani-Muller *et al.* 2000), *Deschampsia caespitosa* and *Anthoxanthum odoratum* (Brookes, Collins & Thurman 1981), *Silene maritima* (Baker 1978) and the hyperaccumulator *T. caerulescens* (Meerts & Van Isacker 1997).

In the field (**Chapter 2**) and under controlled conditions (**Chapter 3 & 5**), the CW population was found to have a significantly higher foliar Zn concentration and concentration factor than GM; that is GM had a relatively lower foliar Zn concentration compared to the total Zn concentration of the soil it was growing in. In the field, the GM soil Zn concentration was two- to three-fold higher than CW and the foliar Zn concentration did not differ significantly between sites. Thus the difference in concentration factor between the two populations could be due to a greater root to shoot restriction as a Zn tolerance mechanism. Baker (1978) concluded that Zn was excluded from the shoots in metallicolous populations of *Silene maritima* as a tolerance mechanism, however the root and shoot Zn concentration and root: shoot ratio varied between metallicolous

populations and could not be ascribed purely to the soil total Zn concentration of the site.

As shown by Barrutia *et al.* (2009), nutrient availability is a limiting factor to shoot biomass and metal tolerance in *R. acetosa*. Therefore in the field, metal tolerance could be linked to the nutrient availability and the solubility of the Zn ions of the growth medium, although it is also probably dependent upon the availability of other metal ions such as Pb and Cd, which are frequently found at mining sites (Baker & Proctor 1990), and the soluble calcium ion concentration and percentage organic matter (Simon 1978).

In conclusion, under the absence of herbivory, the relatively higher foliar Zn concentration of the CW population seems to be due to a lower capacity to restrict Zn uptake into its shoots, compared to the GM population. GM's greater ability to restrict Zn uptake to its shoots seems to be more costly than the tolerance of a higher foliar Zn concentration, as shown by GM's inferior competitive ability compared to CW. This inferior competitive ability is however only seen under competition at moderate Zn contamination over a six month period, potentially due to the size of the plants depleting resources enough for the weaker competitor to be significantly affected. In highly Zn contaminated soils, the small size of the plants reduces the competitive interactions between the two plants and thus the competitive ability has no influence on the competitive outcome between the metallicolous populations of *R. acetosa*.

5.4.2 *Competition under the Presence of Herbivory:*

The second part of this experiment was designed to investigate the same situation but under the presence of herbivory, to determine if herbivory influenced the competitive interactions between the two populations under the same soil Zn treatments. Since the competitive superiority of the CW

population was maintained under the presence of generalist herbivory, null hypothesis 2 was rejected.

Although the presence of generalist *H. aspersa* herbivory caused a reduction in shoot biomass, no significant difference was found in generalist *H. aspersa* preference, between the inter-population-pairings or the soil Zn treatments. The non-significant effect of soil Zn treatment and population identity on percentage damage, suggests that the foliar Zn concentration of both populations of *R. acetosa* grown in the two soil Zn treatments was potentially too high to influence *H. aspersa* preference, even though statistically significant differences in foliar Zn concentration between the soil Zn treatments and populations were found. Foliar Zn concentrations were between 500-600 mg/kg for the 1500 mg/kg Zn plants and between 1200-2200 for the 45,000 mg/kg plants, much higher than non-metallicolous plant concentrations (Broadley *et al.* 2007). Therefore the population differences in foliar Zn concentration did not influence generalist *H. aspersa* herbivore preference, and thus did not affect the competitive dominance of the CW population. This conclusion is supported by the non-significant correlations between foliar Zn concentration and overall percentage damage. Hence for a generalist herbivore, unused to metalliferous sites, small differences in the elevated foliar Zn concentration of metallicolous populations of *R. acetosa* probably do not affect herbivore preference over this experimental timescale, in contrast to the shorter feeding-trials of **Chapter 3**. Thus, although elevated foliar metal concentrations may reduce generalist herbivore damage, as seen in previous laboratory studies investigating the EDH, metallicolous population differences in foliar Zn concentration may not influence generalist *H. aspersa* preference over longer time periods and thus do not affect the potential competitive advantage to metal uptake as an herbivore defence strategy. To confirm that high foliar Zn concentrations are acting as the anti-herbivore trait rather than other unknown factors such as high levels of

secondary metabolites or low nutritional quality, comparisons with *R. acetosa* populations from a less contaminated site is required as a control, to determine if it is high foliar Zn concentrations generally or these unknown factors which are influencing *H. aspersa* preference and the consumption rate.

Additionally, the presence of generalist herbivory did cause an increase in the foliar Zn concentration of the plants, significantly more so at the higher soil Zn treatment, and were more apparent for the GM population at this concentration. The inducement of metals in the foliar tissues from herbivore damage has not been extensively studied. Clipping of plant material has shown to increase foliar Ni concentrations in the hyperaccumulators *Alyssum pintodasilvae* (de Varennes *et al.* 1996) and *Berkheya coddii* (Brooks & Robinson 1998), however these clipping regimes were designed to examine the long-term Ni phytoextraction potential rather than herbivore interactions. However in the Ni hyperaccumulator *Streptanthus polygaloides*, simulated herbivory (through clipping) and herbivory by *Pieris rapae* larvae for eight days did not increase foliar Ni concentration but did induce glucosinolate defence compounds. Additionally clipping of *T. caerulea* did not increase foliar Zn concentrations, regardless of ecotype or Zn concentration or distribution (Haines 2002a). The number of damage- instances as well as the time period may account for the variety of responses to tissue damage in these studies. The latter two studies inflicted one instance of clipping, whereas the first two studies inflicted clipping over a long time period. Thus the potential increase in foliar metal concentrations may require several damage instances before becoming significant. Massey *et al.* (2007) found that silica concentration in grasses did not increase after a single instance of herbivory, but were significantly increased after 16 instances of herbivory. Thus the influence of herbivory on the possible direct or indirect induced effect of foliar Zn concentration remains unknown but potentially requires repeated damage to

investigate it. This may then incur further costs on the plant, influencing its competitive ability.

5.5 Conclusion:

In conclusion, the CW population was the superior competitor for resources overall, as shown by its greater shoot biomass when grown under inter-population competition with the GM population. The presence of generalist adult *H. aspersa* herbivory did not influence the competitive ability of these two metallicolous *R. acetosa* populations, as there was no preference seen for a particular population and therefore the presence of herbivory simply caused a reduction in the biomass of both populations. Although elevated foliar metal concentrations may reduce generalist herbivore damage overall, population differences in foliar Zn concentration and/ or the plants' response to growth in Zn amended soil, do not influence generalist *H. aspersa* preference, and thus do not affect the potential competitive advantage to metal uptake as an herbivore defence strategy. However the soil Zn concentration did however affect the competitive superiority of the CW population. Growth under moderate soil Zn contamination produced larger plants which competed more for the limited resource supply in the pots over a six-month period. Growth under high soil Zn contamination produced smaller plants which competed comparatively less. Hence the competitive superiority of the CW population was masked under high soil Zn contamination growth conditions, as the competition between the two populations was less intense.

Therefore the conclusion reached in **Chapter 4**, of the complimentary, equal competitive abilities of the CW and GM populations, and their potential for competitive co-existence (Aarssen 1984) can be disregarded under a longer period of competition for shared resources. The population differences seen in the competitive ability of metallicolous *R. acetosa* plants gives an indication that

limited resources may have enable the CW population to become either more efficient at capturing resources or require less resources, compared the GM population. Population differences in the foliar Zn concentration and ability to restrict Zn uptake into the shoots could also affect the shoot biomass and hence competitive ability of the metallicolous *R. acetosa* populations. The lower foliar Zn concentration of the GM population compared to CW when grown under a standard Zn –spiked growth medium suggests the GM population is able to restrict Zn uptake into its shoots by a greater degree, which could potentially be more costly to the GM population and thus additionally reduce its competitive ability compared to the CW population. The increased foliar Zn concentration of plants under herbivory in the highly-contaminated soil Zn treatment suggests that repeated or continuous herbivore damage may increase foliar Zn concentration, however this was only significant for the GM population and not seen for the moderate soil Zn treatment.

In conclusion, it appears that population differences in metal tolerance and competitive ability have been influenced more by the soil Zn concentration than by generalist herbivory. As palatability did not differ between metallicolous populations or soil Zn treatments, differences in foliar Zn concentration may not affect the preference of generalist herbivores. It is suggested that the use of a *R. acetosa* population from a less contaminated site should additionally be compared to control for and determine the significance of other potential anti-herbivore traits on *H. aspersa* preference and consumption. Additionally, the influence of herbivores more tolerant to or specialising in the range of foliar metal concentrations present in the metallicolous plant community needs to be tested. Due to the increased foliar Zn concentration seen in herbivore damaged plants in the high soil Zn treatments, the influence of foliar metal concentration on herbivory, and the influence of herbivory on foliar metal concentration also

needs to be untangled to fully support or reject the Elemental Defence Hypothesis.

Chapter 6: General Discussion and Conclusion:

This thesis concentrated on two important ecological processes – plant-herbivore interactions and plant-competition, and investigated their interaction with moderate and highly Zn contaminated soils. This chapter discusses the main findings from the experiments addressing these processes and summarises how the original research presented in this thesis advances our understanding of herbivory and competition in metallicolous plant communities.

6.1 Plant-Herbivore Interactions:

Plant-herbivore interactions are significantly influenced by the quality of the plant food for the herbivore. Plants are thought to be ‘cruddy’ food for herbivores because of their high carbon to nitrogen ratio, sometimes low water content, indigestible compounds and mechanical and chemical defences (Hartley & Jones 1997). Most chemical defences are generally perceived to be carbon-based (Spiteller, Sven Erik & Brian 2008). However, this thesis focused on the relatively novel idea that plants may use inorganic chemical defences as well; specifically it investigates Zn and its effect on plant-herbivore interactions.

6.2 The Elemental Defence Hypothesis:

The Elemental Defence Hypothesis (EDH), first proposed by Boyd & Martens (1992), suggested that elevated metal concentrations in plants can provide protection against natural enemies such as fungi, pathogens and herbivores (Boyd & Martens 1994). The EDH was firstly proposed as a potential advantage of Ni hyperaccumulation, although not necessarily an advantage directly selected for, or the sole advantage (Boyd & Martens 1992). The potential novelty of highly elevated foliar metal concentrations, their lower ‘cost’ in comparison

with the biosynthesis of organic defences, their potential inability to be chemically degraded and their highly toxic effects on natural enemies in both plants and artificial diets, suggests the hyperaccumulation of Ni and metals in general, is potentially a successful plant defence tactic (Boyd & Martens 1994; Boyd 1998). Although experimental tests of the EDH are often supportive, many studies investigating this relatively new hypothesis produce conflicting results, suggesting that more research is required across a broader range of metals, plant families and herbivore guilds, in order to gain a deeper understanding of the influence of metals in plant-herbivore interactions (Boyd 2007).

In a recent review of the EDH, Boyd (2007) found that half of all studies (17 out of 34) investigating the EDH focused on Ni, and these Ni-studies also investigated a broader range of natural enemies and the influence of foliar Ni concentrations on the plant as well as the natural enemy. However only eight studies investigated Zn, and fewer still Se, Cd and arsenic (As). Additionally the vast majority of the plants studied were hyperaccumulating plants, and for Zn studies, only three plant species have been tested, the most popular being *Thlaspi caerulescens*. This thesis contributed to the limited numbers of studies involving Zn by investigating how Zn accumulation affects the palatability of the non-hyperaccumulator *Rumex acetosa*, a species which takes up high levels of Zn, with potential consequences for its susceptibility to herbivory and competitive ability, but not to such an extent as hyperaccumulating species.

6.2.1 *Using a Variety of Experimental Approaches:*

Different experimental approaches are required to fully understand the influence of metal-accumulation on plant-herbivore interactions. For instance, some of the studies have manipulated the foliar metal concentration either

through the soil metal concentration (e.g. Martens & Boyd 1994; Pollard & Baker 1997; Boyd & Martens 1999), or by comparing related hyperaccumulator and non-hyperaccumulator plants species (Boyd & Moar 1999; Davis & Boyd 2000). This gives us valuable information on the influence of soil metal concentration on the palatability of a plant species; and the palatability of hyperaccumulating and non-hyperaccumulating plants found on metalliferous soils. Nevertheless, the actual cause of the anti-herbivore trait(s) is confounded by the plant's response to the soil metal concentration and between-species differences in other plant defences such as organic compounds (Boyd & Moar 1999; Davis & Boyd 2000; Huitson & Macnair 2003; Macnair 2003). This confounding effect was seen when comparing plant damage within- and between a hyperaccumulator and non-hyperaccumulator in the field (**Chapter 2**). The five-fold higher foliar Zn concentration and two-fold lower shoot damage of *T. caerulescens* compared to *R. acetosa* in the field indicated that between-species differences in foliar Zn concentration may affect herbivore damage, despite possible confounding effects. However, the variation in foliar Zn concentration within a site was not correlated with the shoot damage for either species. Thus, finer-scale within-species differences in elevated foliar Zn concentrations do not seem have a strong effect on herbivore damage in the field, and therefore within-species differences in herbivore damage could be due to the influence of site on other palatability aspects.

An alternative approach is to take advantage of within-population genetic variation in foliar metal levels and test the impact of such variation on herbivore preference (Jhee *et al.* 1999). Additionally, one study (Boyd *et al.* (2002) has used between-population genetic variation to compare herbivory experienced by hyperaccumulating and non-hyperaccumulating populations of *Senecio coronatus* found on metalliferous soils. Metallicolous and non-metallicolous populations of *T. caerulescens* (from continental Europe) have also

been used to investigate the influence of Zn on palatability, although the influence of glucosinolates in a potential trade-off (Noret *et al.* 2005) and other life-history differences (Dechamps *et al.* 2007; Dechamps *et al.* 2011), may produce confounding factors on palatability. The comparison of two metallicolous populations from different sites within one region, a technique used in this thesis, is therefore another way of investigating how foliar metal concentration influences plant palatability without producing potentially considerable confounding differences in other plant traits. However, knowledge of potential between-population differences in palatability, such as the carbon to nitrogen ratio, macronutrient concentration and organic defences, is required for a full understanding of plant palatability in this system. As these plants traits may be genetically linked to others, and thus not independent of metal tolerance or growth in contaminated soils, Boyd (2007) suggests that future studies should combine artificial diet studies with investigation into how genetic variation in metal accumulation affects herbivore preference and performance. Artificial diets have their own problems however; they need to consider the chemical form of the metal element in the plant and the plant's nutritional status, in order to simulate the true effect of metal-accumulation on plant palatability (Trumble, Kund & White 1998; Vickerman & Trumble 1999).

6.2.2 *Using Lower and Less-Contrasting Foliar Metal Concentrations to Investigate the EDH:*

The use of metallicolous plant species and populations with lower 'accumulator' concentrations of metals rather than hyperaccumulating concentrations, would allow the investigation of whether this lower concentration range still provided a defensive function (Boyd & Moar 1999; Boyd 2007). Many ecotoxicological studies have reported negative effects of metals on herbivores in no-choice artificial diets. These ecotoxicological studies

suggest that dietary Zn concentrations much lower than the 10,000 mg/kg hyperaccumulator threshold affect the consumption behaviour and growth of *Helix aspersa* (Laskowski & Hopkin 1996b; Gomot-De Vaufléury 2000; Chevalier, Le Coz-Bouhnik & Charrier 2003) and other chewing generalist herbivores (Coleman, Boyd & Eubanks 2005; Notten *et al.* 2006a; El-Gendy, Radwan & Gad 2011). Additionally, rather than the highly contrasting diet choice of a hyperaccumulator compared with a non-accumulating plant, smaller differences in the foliar metal concentration may still have an impact on herbivore preference and performance (Huitson & Macnair 2003). Therefore, to fully understand the function of accumulated elements and their impact on herbivory, the potential for differences in relatively low metal concentrations to affect herbivory, known as 'defensive enhancement', is required (Boyd 2007).

This thesis used metallicolous populations of the non-hyperaccumulating plant *R. acetosa*, to investigate the effect of foliar Zn concentrations that are <10,000 mg/kg (i.e. below the designated hyperaccumulator threshold) on plant palatability. Most plant species found on metalliferous sites have elevated metal concentrations, for instance the Zn concentration is usually 50-500 mg/kg in the shoots of plants growing on Zn-rich soils (Reeves & Baker 2000). However metallicolous populations of *R. acetosa* were found to have highly elevated foliar metal concentrations, ranging from 500-8000 mg/kg (over all study conditions). Although these foliar Zn concentrations are not at hyperaccumulating levels, foliar Zn concentrations of greater than 3000 mg/kg still deserve attention (Boyd & Moar 1999; Reeves & Baker 2000) and are likely to be ecologically relevant.

To investigate the influence of non-hyperaccumulation levels of foliar Zn concentration on herbivore preference (without the confounding influence of species differences) two metallicolous populations of *R. acetosa* were used (**Chapter 3**). By manipulating the foliar Zn concentration through the soil Zn

concentration, leaves with approximately 1250 and 3500 mg/kg foliar Zn concentrations were offered to *H. aspersa*, i.e. a contrast of approximately 2000 mg/kg Zn. Plants from two different populations grown in a low soil Zn treatment differed in their foliar Zn concentrations by approximately 200 mg/kg, whereas plants from those populations grown under high soil Zn differed by approximately 700 mg/kg. Leaves from these different populations and soil Zn treatments were offered to *H. aspersa*, but after 72 hrs., less than 35% of any of the leaves presented had been consumed. Preliminary studies using metallicolous and non-metallicolous populations of *R. acetosa* grown in non-Zn amended growth medium, had shown that *H. aspersa* will eat a large percentage of the leaf (data not shown: foliar Zn concentration \approx 75 mg/kg, approximate percentage consumed over 24 hrs. \approx 5–100 %), potentially indicating that this magnitude of foliar Zn concentrations in *R. acetosa* deterred excessive herbivory. As no difference in percentage damage was seen between plants grown in the two different soil Zn treatments, it is suggested that *H. aspersa* (with no previous known experience of metalliferous sites) may not be able to distinguish between the foliar Zn concentration choices offered to it. Thus a foliar Zn concentration of greater than 1000 mg/kg in *R. acetosa* could be enough to deter *H. aspersa* herbivory generally, and it may not be able to distinguish between foliar concentrations higher than this. As the only significant difference in palatability was found when comparing the leaves from the two populations when grown in the high soil Zn treatment, it seems that palatability is influenced more by population differences in response to growth in high soil Zn concentrations, than by the differences in the foliar Zn concentration offered.

As no other studies have looked at the EDH in *R. acetosa*, direct comparisons cannot be made. However two other studies have investigated the effect of foliar Zn on the preference on *H. aspersa*. Noret *et al.* (2005) investigated the preference of *H. aspersa* with *T. caerulescens*, and also manipulated the foliar Zn

concentration through the soil Zn concentration and population differences. Within-population trials also showed no significant difference in consumption between soil Zn treatments; here *H. aspersa* was presented with foliar Zn concentrations of approximately 2600 and 5200 mg/kg. Between-population trials also used choices which did not differ significantly in their foliar Zn concentration, but here *H. aspersa* preferred the metallicolous populations over the non-metallicolous populations. The lower glucosinolate concentration of the metallicolous populations may have made these leaves more preferred, even though they also generally contained higher foliar Zn concentrations. In another study, Huitson & Macnair (2003) offered *H. aspersa* F₂ plants from crosses between *Arabidopsis halleri* and *A. petraea*, grown under different growth medium Zn concentrations. Again the plants in the choice test did not significantly differ in their foliar Zn concentration, which was approximately 2000 mg/kg. However, *H. aspersa* consumption was deterred, not by higher foliar Zn concentrations *per se*, but from growth in the higher Zn growth medium levels. Thus the foliar Zn concentrations used in these studies may either be too high for the generalist *H. aspersa* to distinguish between in short-term feeding trials, or it is possible that *H. aspersa* preference is more influenced by other plant attributes, such as nutritional content or organic/ organometallic compounds (Huitson & Macnair 2003; Noret *et al.* 2005).

6.2.3 *Metal Accumulation and its Interaction with Other Plant Defences:*

Plants have a vast array of different physical, chemical and signalling defence mechanisms, so it is unlikely that a particular defence operates in isolation – its impact will depend on which other defences are active against the herbivore and all defence mechanisms may be influenced by other factors, such as the nutritional status of the plant. Thus to fully understand the ecological significance of a particular plant defence, it must be placed in context with the

others (Agrawal & Fishbein 2006; Rasmann & Agrawal 2009). In the 'trade-off' hypothesis, the presence of lethal foliar metal concentrations may reduce the levels of other more 'costly' organic defences, potentially benefiting the plant by reducing the cost of defence overall (Boyd & Martens 1998b; Boyd 2007). This potential trade-off has been studied between hyperaccumulating and non-hyperaccumulating plant species (Davis & Boyd 2000), through soil manipulation of the foliar metal concentration (Tolrà *et al.* 2001; Jhee *et al.* 2006) and through differences between metallicolous and non-metallicolous populations (Noret *et al.* 2005). However, all these studies have only investigated this interaction with one type of organic defence compounds – the glucosinolates. In the Ni-hyperaccumulator, *Streptanthus polygaloides* (Brassicaceae), Davis & Boyd (2000) found that the total glucosinolate concentration was lower than that of a related non-hyperaccumulator, but this did not interact with the soil Ni concentration. They suggested that the potential trade-off between glucosinolate and Ni hyperaccumulation may be constitutive and thus not affected by changes in the soil conditions. Noret *et al.* (2005) investigated the effect of total foliar Zn and glucosinolate concentration on the palatability of *T. caerulescens* to *H. aspersa*. The foliar Zn concentration did not universally reduce the plant palatability, as the higher glucosinolate concentrations found in non-metallicolous populations seemed to have more of a negative effect on palatability. The complexity of their results may be due to potential population differences in glucosinolate composition and their response to growth in contaminated and non-contaminated soils. In *T. caerulescens*, Tolrà *et al.* (2001) found that glucosinolate concentration was affected qualitatively and quantitatively by the growth medium Zn concentration, and that a potential trade-off occurred between glucosinolate and Zn concentrations in the shoots of *T. caerulescens*, but not in the roots.

The plant's response to soil metal concentration is important when considering the impact of metal-accumulation on plant-herbivore interactions. Jhee *et al.* (2006) found that *S. polygaloides* plants grown in low-Ni soils produced more allyl glucosinolate (sinigrin), whereas those in high-Ni soils produced more 4-OH-methylindolyl. The presence of sinigrin was found to influence oviposition of *Plutella xylostella* more so than the presence of Ni. As a Brassica specialist, the larvae were able to circumvent the glucosinolate defence chemicals in the low-Ni plants, and decrease the plants' biomass and reproductive fitness. Although the increased foliar Ni-concentration of plants grown in the high-Ni-soil treatments did not affect oviposition rates, they were found to produce a novel defence against larvae feeding, confirmed by artificial diet experiments.

Behmer *et al.* (2005) suggest that the locust *Schistocera gregoria* may not be able to taste Zn directly in *T. caerulescens* plants or synthetic food, as choice trials showed no difference in the feeding period of the first meal. However, in trials lasting several days, a preference for low-Zn plants and synthetic food was seen, suggesting a post-ingestive feedback mechanism. In subsequent trials, locusts were able to learn to associate the presence of coumarin with Zn in their diets, and rejected food containing coumarin but no Zn. This provides further indication that generalist herbivore dietary preference of metal-accumulating plants is dependent upon detecting differences in both the plants' organic and inorganic chemistry.

The above studies show an interaction between elemental and organic defences and support the 'trade-off' hypothesis, demonstrating the importance of studying plant defence using a variety of genetic, biochemical and ecological methods and in context with other plant traits and defences (Rasman & Agrawal 2009). For example, plants may (hyper)accumulate several different metals to varying degrees (Reeves & Baker 2000; Schat, Llugany & Bernhard

2000; Macnair 2003). For example *T. caerulescens* hyperaccumulates Zn and Cd, but population and ecotypic differences are sometimes seen in Cd tolerance and accumulation (Baker, Reeves & Hajar 1994; Lombi *et al.* 2000; Zha *et al.* 2004). Zha *et al.* (2004) suggests that Zn and Cd accumulation in *T. caerulescens* is regulated by multiple genes and that the metal transport system is capable of transporting multiple metals. However the effect of multiple metals, and both metal and organic defence compounds on herbivore palatability has only been recently studied by Jhee, Boyd & Eubanks (2006). When pairing Zn with either Ni, Pb or Cd, and when pairing Ni with either tannic acid, atropine or nicotine, the combined effect on herbivore (*Plutella xylostella*) survival or pupation was generally significantly greater than the single effect. Thus synergistic interactions between accumulated metals and prevalent organic defence compounds may make accumulated metals more potent to a range of herbivores and elemental defence more widespread than previously thought (Jhee, Boyd & Eubanks 2006; Boyd 2007). Thus, when considering plant palatability in plant-herbivore interactions, it is more ecologically relevant to think of a suite of traits, including nutritional quality, physical defences (e.g. spines and leaf toughness), organic defences (e.g. tannins and alkaloids), regrowth capacity (i.e. tolerance) and indirect defences (e.g. signalling volatiles and parasitoids) (Agrawal & Fishbein 2006), as well as non-organic defences (e.g. Zn and Si). The EDH has generally focused on metal-accumulation as a deterrent to herbivory and its lethal effects. However metal accumulation may affect other plant responses to herbivore damage. Palomino *et al.* (2007) suggest that soil Ni may enhance the compensatory re-growth (i.e. tolerance) of *Thlaspi montanum* to intense plant damage. When 50% artificial damage was applied, the reduction in growth was affected by the soil Ni concentration. Plants grown without Ni had a greater reduction in their stem diameter, compared to plants grown with Ni. However this interaction was not seen at lower levels of artificial damage.

Additionally, other plant herbivore defences may also be affected by metal accumulation. Certain 'defence' traits are also known to have other adaptive advantages, for instance leaf trichomes have potential anti-herbivore and water-loss functions (Woodman & Fernandes 1991). In tobacco plants (*Nicotiana tabacum*), trichomes secrete organic compounds such as diterpenes, and were recently discovered to increase in abundance and secrete high quantities of calcium (Ca) and Cd from them as well, possibly as a tolerance mechanism to high Cd concentrations (Choi *et al.* 2001; Harada & Choi 2008). Aside from the EDH, Boyd & Martens (1992) presented four other potential advantages for metal hyperaccumulation in plants found growing on metalliferous soils: **(i)** increased metal tolerance by complexing excess metals in the shoots; **(ii)** increased drought resistance; **(iii)** a potential competitive interference/allelopathy response through the disposal of metal-rich leaves and so increasing the metal concentration around the plant; and **(iv)** inadvertently through increased water and nutrient uptake efficiency. These potential advantages are little-studied (Boyd 2004), and consequently elevated foliar metal concentrations may be the result of other abiotic adaptations, as well as potentially providing a benefit in biotic interactions.

6.3 Plant-Competition:

As well as being influenced by herbivores, plants are also influenced by the presence of other individuals, especially so if essential resources such as light, water or nutrients are limited in supply. The competitive ability of a plant to acquire essential resources for growth, defence and reproduction is another important ecological process that influences the interactions between a plant and its abiotic and biotic environment.

Metalliferous soils generally have a low nutrient and water status, and are especially deficient in nitrogen, phosphorus and other macro-nutrients , exacerbated by nutrient leaching (Smith & Bradshaw 1979; Ye *et al.* 2002). Elevated metal concentrations in the soil produce toxic effects in plants, even in those with specialised physiological processes in place to tolerate the abiotic stresses and to restrict metal uptake (Baker 1987; Macnair 1997). When grown in non-contaminated soils, metal-tolerant plants are generally smaller with lower reproductive potential and competitive ability, compared to their non-tolerant counterparts (Cook, Lefèbvre & McNeilly 1972; Hickey & McNeilly 1975; Wu 1990). Processes conferring metal tolerance are thought to incur metabolic costs at the expense of productivity and reproductive output, i.e. there is a potential trade-off between metal tolerance and fitness (Baker 1987; Ernst, Schat & Verkleij 1990; Wu 1990; Harper, Smith & Macnair 1997a; Kazakou *et al.* 2008; Maestri *et al.* 2010). However, there is a potential competitive advantage to metal uptake as higher shoot metal concentrations may protect the plant from herbivory due to deterrence and reduced herbivore performance (Boyd & Martens 1992). The costs of metal uptake in terms of growth may be outweighed by the benefits to growth gained through protection against herbivory; thus the competitive ability of plants in metalicolous communities will reflect their ability to uptake the resources required for both survival and defence.

Potential population differences in competitive ability were investigated in this thesis through inter- and intra-population competition experiments at a constant total number of plants per pot. This thesis showed that soil Zn concentration influenced the degree of competition between plants, and thus interacted with competition type (**Chapter 4**) and population identity (**Chapter 5**) to influence a plant's competitive ability to acquire resources. Decreased growth under higher soil Zn concentrations reduced the competitive

interactions between plants. A longer experimental time period showed that population differences in competitive ability were seen at moderate soil Zn concentrations, but not at higher soil Zn concentrations (**Chapter 5**). Inter-population competition produced larger plants under moderately contaminated levels of soil Zn, whereas intra-population competition produced larger plants under highly contaminated levels of soil Zn (**Chapter 4**). Thus, the population differences in competitive ability found in **Chapter 5** could have driven the interaction between competition type and soil Zn concentration seen in **Chapter 4**, to influence the competitive ability of metallicolous *R. acetosa* populations. This may link to the differences in the foliar Zn concentration of different populations grown in the same soil Zn concentration, and to potential population differences in the resources required for metal tolerance. However, due to reduced plant growth and thus reduced competitive interactions between plants, competition under low densities within a species, may not play a major role in influencing plant growth at high soil Zn concentrations.

The adverse effects of the high metal concentrations found on bare spoil heaps on plant growth have been well documented in studies which also provide evidence for natural selection of metal tolerance in plants which are able to grow there (Antonovics, Bradshaw & Turner 1971; Baker 1987). More recently, the effects of moderate soil Zn contamination produced by erosion of galvanised electricity pylons have been studied. In this system, a relatively weaker selective pressure for metal tolerance compared to mining sites, but a greater tolerance compared to uncontaminated soils, has been seen (Al-Hiyaly, McNeilly & Bradshaw 1990). However, the density of vegetation present on heavily contaminated soils may influence the competitive ability of that population: the low density of vegetation on bare spoil heaps suggests that these populations may be under lower selective pressure for traits associated with resource competition, but under much stronger selection for traits

associated with metal tolerance (Nicholls & McNeilly 1985). However, the less toxic effects of moderately contaminated soils would allow colonisation by a greater abundance and taxa of plants and therefore selection for competitive ability may be more intense, whilst selection for tolerance traits may be relatively weaker (McNeilly 1968). Therefore competitive ability may be tightly linked to metal tolerance on highly contaminated sites, whereas on moderately contaminated sites competitive ability will reflect interactions between competition for resources and metal tolerance.

6.4 The Influence of Herbivory on Plant Competition:

Herbivory and competition do not act alone on plants and herbivory is well known to influence the competitive ability of a plant (Crawley 1997). Herbivory often decreases the growth and fecundity of a plant, and may even cause mortality, but it can also stimulate regrowth as a tolerance mechanism (Strauss & Agrawal 1999). It may also alter the root: shoot ratio of plants, thus by altering the plant's relative ability to require limited soil nutrient and water resources, and preferential feeding by herbivores on one plant as opposed to a competing one, can shift the competitive balance in favour of the less palatable one e.g. (Bentley & Whittaker 1979; Rodriguez & Brown 1998; Strauss & Agrawal 1999; Schädler, Brandl & Haase 2007). By such mechanisms, herbivory is an important influence on the outcome of competitive interactions between individual plants and different plant species (Louda, Keeler & Holt 1990).

The presence of generalist adult *H. aspersa* herbivory did not influence the competitive ability of the two *R. acetosa* metallicolous populations used in this thesis, as there was no preference for one population over another and so no difference in foliar damage rates between populations (**Chapter 5**). Therefore the presence of herbivory simply caused a reduction in the biomass of both

populations, thus maintaining the competitive superiority of the Clough Wood population seen throughout this thesis. Predicting the competitive outcome if a preference had been seen is difficult, because of the interaction of soil Zn concentration with plant palatability (**Chapter 3**) and competitive performance (**Chapter 4**). **Chapter 3's** results suggest that *H. aspersa* may have initially preferred the Gang Mine population over the Clough Wood population, especially for the 45,000 mg/kg soil Zn treatment. However, **Chapter 4's** results indicate that at this soil Zn treatment, the *R. acetosa* plants are too small for population differences in competitive ability to be detected, and thus competition does not seem to be a great influence under these experimental conditions. Thus, the low consumption and lack of preference, of generalist herbivores from non-metallicolous communities such as *H. aspersa*, seems unlikely to influence the within-species competitive interactions between individual plants on highly contaminated sites. The increase in the foliar Zn concentration of the Gang Mine population under the presence of herbivory seen in **Chapter 5**, does suggest that herbivory may influence the foliar Zn concentration of *R. acetosa* over a longer time period. As temporal patterns in herbivore preference and plant growth rate were not investigated in this thesis, the influence of long-term herbivory on the competitive interactions of metal-tolerant plants is unknown. Additionally, competitive interactions in metallicolous communities may be additionally influenced by the preference of specialist herbivores and the density of the vegetation, as well as interactions with other plant species.

6.5 Conclusions and Areas for Further Research:

6.5.1 *General Conclusions:*

This thesis has shown that Zn accumulation has an important influence on plant-herbivore interactions and plant competitive ability, and has also highlighted several areas which require further research to fully understand these interactions. This thesis has shown that Zn accumulation in the non-hyperaccumulator *R. acetosa* has important consequences for both plant-herbivore interactions and the competitive ability of plants in metallicolous populations. Whilst the accumulation of foliar Zn in *R. acetosa* prevented excessive consumption of the leaves in all experiments, generalist *H. aspersa* preference seems to be more influenced by differences in how plant populations respond to growth in Zn contaminated soil, rather than the foliar Zn concentration *per se*. Additionally a field study indicated that species-differences, but not within-population variation, in foliar Zn concentration affected herbivore damage. Although this thesis provides some support for the EDH, the interaction between metal-accumulation and other plant defences needs to be investigated further to fully understand the extent of elemental defences in plant-herbivore interactions. Competitive interactions between metallicolous populations of *R. acetosa* were significantly affected by the soil Zn concentration and may relate to potential population differences in resource requirement for metal tolerance. However, due to reduced plant growth and thus reduced competitive interactions between plants, within-species competition under low densities may not play a major role in influencing plant productivity at high soil Zn concentrations. Thus further work investigating the competitive ability of metal-tolerant plants, should also investigate the influence of plant density. The presence of the generalist herbivore *H. aspersa* did not affect the competitive ability of the metallicolous plant populations, possibly due to the lack of metal tolerance in this herbivore species, which

meant they were unable to consume sufficient foliage to influence the outcome of the competitive interactions. Suggestions for further investigations and improvements into the *Rumex acetosa* – *Helix aspersa* study system used in this thesis, and investigation into elemental defence and the joint effects theory are now described below.

6.5.2 *Areas for Further Research:*

6.5.2.1 Herbivore Type:

Although *H. aspersa* has been widely used in metal ecotoxicological studies and in testing the EDH due to its widespread distribution, generalist diet and culturing ease, this species may not always be a useful model organism to investigate the role of Zn in plant-herbivore interactions. As *H. aspersa* is not restricted to metalliferous habitats, high foliar metal concentrations present a novel deterrence, potentially indicated in this thesis by the low feeding rates of *H. aspersa* collected from non-metalliferous sites on Zn-rich leaves. However, pilot trials using cultured *Mamestra brassicae* (Cabbage Moth, Noctuidae), another widespread herbivore with a broad diet, also did not show any effect on foliar Zn concentration on the percentage consumed in preference trials, although in many cases the pairs of leaves were completely consumed within 24 hrs. instead. Additionally, a low number ($n \approx 20$) of the yellow-banded snails *Cepaea hortensis* and *C. nemoralis* were collected from Gang Mine in 2009, sheltering under large stones, however these individuals were found not to eat fresh plant material (metalliferous or non-metalliferous), but instead preferred decaying plant material. Thus behavioural feeding responses such as the sensitivity of the herbivore to foliar Zn concentration and the time-scale required for feeding preferences to become established, as well as the influence of metal toxicity on consumption rate, herbivore growth rate and thus total foliage consumption, will additionally play an important role in the outcome of

feeding trials (see Noret *et al.* 2007a). Additionally, few studies have documented the extent of herbivory in metalliferous sites (Noret *et al.* 2007b; Galeas *et al.* 2008) or investigated plant-herbivore interactions using herbivores adapted to feeding on plants from metalicolous communities (Boyd & Wall 2001; Schwartz & Wall 2001; Boyd *et al.* 2006; Freeman *et al.* 2006). Population differences in the Pb concentration of *H. aspersa* from metal-contaminated and non-contaminated sites have been found for the soft tissues (Beeby & Richmond 1987) and shell to soft tissue ratio (Newman *et al.* 1994), and also for juvenile shell mass (Beeby, Richmond & Herpé 2002), suggesting genetic and phenotypic adaptation in metal-tolerant populations of *H. aspersa* through the modification of the calcium metabolism and incorporation of Pb into the shell rather than the soft tissues (Beeby, Richmond & Herpé 2002). Population differences in the Pb, Zn and Cd concentration of the soft tissues of the terrestrial slugs *Arion subfuscus* and *Deroceras reticulatum* from metalliferous and non-metalliferous sites have also been found, although this interacted with season (Greville & Morgan, 1991). This suggests that population differences in metal tolerance and accumulation could occur for a variety of molluscan herbivores found at metal-contaminated sites. Thus, it is suggested that future studies consider using generalist herbivores from metalicolous plant communities wherever possible to fully investigate the influence of metal uptake on plant-herbivore interactions. Furthermore, further investigations into this thesis's study system should use specialist Polygonaceae and Brassicaceae chewing herbivores, such as the small copper butterfly *Lycaena phlaeas* (Lycaenidae) and the small white butterfly *Pieris rapae* (Pieridae) respectively, which are tolerant of the antiherbivore secondary plant compounds present in these two plant families. Specialist herbivores may offer insight into not just the effects of foliar metal concentration on palatability, but additionally the interaction between elemental and organic defence compounds and the combined role they play in elemental defence.

6.5.2.2 Herbivore Feeding Guild:

Additionally, herbivore feeding guild will play an important role in the effectiveness of elemental defence and the joint effects theory. Studies examining the EDH have mainly used folivores especially leaf-chewing herbivores, which have generally shown an aversion to high foliar metal concentration (Boyd 2007). Although not extensively studied, the accumulation of metals generally occurs in the vacuoles of the epidermal and mesophyll cells of hyperaccumulating plants, as seen for Zn (Küpper, Zhao & McGrath 1999; Ma *et al.* 2005) and Cd in *T. caerulescens* (Ma *et al.* 2005) and for Ni bound with citrate and histidine in *T. goesingense* (Krämer *et al.* 2000). Thus the intra-cellular distribution of metals within hyperaccumulators seems to limit the effectiveness of elemental defence towards leaf-chewers and some cell-disrupter feeding guilds (Jhee *et al.* 2005; Jiang *et al.* 2005), but not phloem-feeders on Ni-hyperaccumulators (Boyd & Martens 1999), although phloem feeders on a Se hyperaccumulator were deterred (Hanson *et al.* 2004). Thus, the effects of foliar metal deposition and feeding guild on the effectiveness of elemental defence need further study over a variety of accumulating and hyperaccumulating plants to confirm this. Studies investigating the EDH using a variety of feeding guilds will enable us to understand if the physiological effects of metal tolerance, accumulation, and tissue metal localization produces corresponding consequences in plant mechanical defences, nutritional quality and secondary metabolites.

6.5.2.3 Contrasting Metalliferous Sites:

Additional comparisons with populations from less contaminated sites such as from Zn galvanised electricity pylons (see Al-Hiyaly, McNeilly, & Bradshaw 1990), rather than from non-metalliferous sites as a control, and manipulation of resource availability to levels found in these field sites, could amplify

population differences in metal tolerance, herbivory and competitive ability, and thus provide more insight into the mechanisms involved. Wherever possible these further studies should also consider using non-hyperaccumulating plants, as they are an important part of metallicolous plant communities and provide insight on how metal tolerance and accumulation affect ecological processes and potentially select for significant plant traits.

6.5.2.4 Clonal Material:

The use individuals grown from seed in the experiments presented in this thesis, did present a high degree of within-population variation in foliar Zn concentration and shoot biomass (especially seen in **Chapter 4 and 5**). As both *R. acetosa* and *T. caerulescens* can reproduce vegetatively through ramets (seen in the field and greenhouse), feeding trials and density-dependant experiments using clonal material will give greater insight and control into the plant traits involved in defence and competitive ability, and the effects of the environment on them. The use of *R. acetosa* and *T. caerulescens* genets in hydroponic experiments can be used to further determine the mechanistic effects of metal tolerance on plant secondary metabolites, such as the organic acids malic acid, oxalic acid and citric acid and phenolic acids in *Rumex* spp. (Mathys 1977; Schöttelndreier *et al.* 2001; Tolrà *et al.* 2005) and glucosides in *T. caerulescens* (Mathys 1977). This could allow the selection of contrasting genetic lines and ecotypes to allow the mechanistic effects of Zn accumulation on plant palatability and competitive ability to be examined in more detail in the lab and the field (Harper, Smith & Macnair 1997; Strauss *et al.* 2002; Deschamps *et al.* 2008; Kazakou *et al.* 2008; Maestri *et al.* 2010).

6.5.2.5 Bioavailable Zinc Fraction and Resource Parameters:

Lastly but not least, the bioavailability of Zn and other metals such as Pb and Cd in the field sites examined should also be considered. Although using the total Zn concentration allows comparison of the Zn concentrations between sites and studies, it does not inform us of the actual concentration available to and taken up by the plant. This is of less concern in the greenhouse studies, where the ZnO used was confirmed to be homogenously supplied in the pots and the pH of the growth medium batches was found to be similar between batches and to the pH of the field sites (ZnO data not shown; pH \approx 5.7), and thus would not have contributed to any between- or within-population variation seen. However in the field metal-metal interactions and metal bioavailability are highly important factors, as the heterogeneity and bioavailability of Zn will be greatly affected by site differences in the Zn species and compounds present, soil pH, total and soluble organic matter content, phosphate concentration, and presence of chelating agents such as citrate and oxalate amongst other factors (Lindsay, 1972; Simon 1978; Meerts *et al.* 2003; Broadley *et al.* 2007). Using a variety of metal extraction techniques such as MgCl₂, acetic acid, ammonium-EDTA and DTPA (Allen 1989; Wong 2003) to determine the Zn soluble fraction and thus estimate the Zn bioavailable fraction will give a clearer picture of the Zn concentration and forms that the metallicolous *R. acetosa* populations are interacting with and potentially adapting to. Analysis of root chemistry and exudates in field reciprocal transplantation experiments and through manipulation of the nutrient concentration and availability in hydroponic experiments (Schöttelndreier *et al.* 2001; Tolrà *et al.* 2005) could provide further information on between-population differences in root chemistry for metal tolerance and nutrient uptake (Punz & Sieghardt 1993). This would potentially allow the proposed trade-off between metal tolerance and competitive ability to be investigated in

more detail (Wilson 1988; Deschamps *et al.* 2008; Kazakou *et al.* 2008). Other further field soil measurements include resource availability for the competition experiments, such as macronutrient and micronutrient concentrations, soil moisture content and soil water holding capacity and light availability and intensity (Kazakou *et al.* 2008). Measurement of these parameters alongside plant fitness ones, such as reproductive effect, relative growth rate, and the root: shoot ratio (Wilson; 1988; Audet & Charest 2008), will allow the cost of metal tolerance and growth on metal-contaminated sites to be reconciled with site differences and species and population differences in metal tolerance (Kazakou *et al.* 2008; Maestri *et al.* 2010).

6.5.2.6 Summary:

In summary, further investigation into this study system using metallicolous herbivore populations and specialist herbivores from a variety of feeding guilds, will provide further insight into the plant-herbivore interactions involved in elemental defence. Comparison of populations from less contaminated sites and using clonal material in feeding trials and density-dependant competition experiments, will allow the effects of zinc accumulation on plant physiological responses such as metal tolerance, secondary metabolite production and resource requirements to be fully investigated. Lastly, the abiotic site factors such as the presence of other metals and their bioavailability, as well as resource availability must also be considered when investigating the ecological consequences of metal accumulation in plants.

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